A CONTRIBUTION TO THE STUDY OF THE OPSONINS.*

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Through the researches of Wright and Douglas[1] it has been established that the phagocytic effect of human leucocytes upon certain bacteria is essentially dependent upon the presence in the blood plasma and serum of substances which they have termed opsonins. They could demonstrate that these substances *per se* are to a certain extent at least thermolabile, being partly destroyed by heating the blood serum to a temperature of 60-65° C. for ten minutes or more. The phagocytic effect of such serum is accordingly diminished and may indeed be suspended. If, however, bacteria are first digested with normal serum and subsequently exposed to a temperature of 60–65° C. phagocytosis proceeds in a normal manner. The organisms in question are the staphylococci, Bacillus pestis, Micrococcus melitensis, Diplococcus pneumoniæ (Fränkel), Bacillus coli, Bacillus dysenteriæ, Bacillus anthracis, Bacillus typhosus, Bacillus tuberculosis and the vibrio of Asiatic cholera, while the diphtheria bacillus and Bacillus xerosis were found to be insensible to opsonic action. With the latter phagocytosis proceeds as promptly with heated as with unheated serum.

In a series of further studies Wright and his collaborators then illustrate the variations in the opsonic content of the blood in certain infections and discuss the significance of the data obtained from the standpoint of immunity. The therapeutic inferences to which they are led and the practical results which

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[1] Wright and Douglas, *Proc. Royal Soc.*, 1903, lxxii, 357; *ibid.* 1904, lxxiii, 128; *ibid.*, 135, and *ibid.*, 147.

they claim to have obtained are highly suggestive and have attracted wide-spread attention.

The studies described in the present paper were undertaken with the idea of gaining a further insight into the nature of the opsonins and their significance from the standpoint of immunity.

TECHNIQUE.

For the purpose of estimating the phagocytic power of the leucocytes Wright makes use of a modification of the method originally devised by Leishman. To this end a sample of blood is drawn off and mixed with one tenth of its volume of a ten per cent. solution of sodium citrate. A second sample of blood is allowed to clot in the usual way. In the case of the first sample the corpuscles are isolated from the plasma by repeated washing with physiological salt solution and centrifugation; washed corpuscles are thus obtained. In the case of the second sample the serum is simply separated from the corpuscles in the ordinary way by centrifugalization. Equal parts of washed corpuscles (two or three) and serum are then taken and further mixed with one volume of a suspension of the organism under consideration, obtained by rubbing up in physiological salt solution a portion of a twenty-four hours growth on agar. The resultant mixture is incubated at blood heat for fifteen minutes, when smears are made and stained with Leishman's stain. Finally the number of ingested bacteria is counted in a series of polynuclear leucocytes, taken in the order in which they are observed, and the average for one leucocyte calculated; the resultant figure denotes the phagocytic power. This is further compared with that of a normal control person, which latter serves as unit. The ratio between the two values is termed the opsonic index of the blood.

This method has thus far been employed almost exclusively in the study of the opsonins, and upon it the results obtained by Wright and his co-workers are based.

In some of the earlier work which was carried on by one of us (Simon) before the present studies were undertaken, the original method of Leishman was used. To this end equal

parts of blood and bacterial suspension are mixed and a drop of the mixture placed on a slide, covered with a coverglass and incubated at 37° C.; slide and cover are drawn apart and the resultant smears stained as above. The average number of organisms pro leucocyte is then determined as with Wright's method. For purposes of class demonstration of phagocytosis this method was found quite satisfactory, but it does not appear to us that either in the original form or in that now employed by Wright the mere determination of the average number of organisms pro leucocyte gives an adequate idea of the quantity of opsonins present. It is thus quite conceivable that in a given case the use of concentrated serum may show what would appear to be a perfectly normal and sufficient quantity of opsonins for a given number of organisms, which amount, however, might be altogether inadequate for a larger number. This inadequacy would become manifest at once upon diluting the serum, when a rapid exhaustion of the phagocytic power would occur. Our own investigations have shown conclusively that such an event is by no means uncommon. Serum of the pig in concentrated condition thus manifests a most intense opsonic effect for staphylococci, which diminishes with marked rapidity, however, on dilution, while with human serum the dilution can in most cases be carried much further, even though the initial phagocytic power is less. Similarly we find that with certain organisms, such as the anthrax bacillus, the colon bacillus, Fränkel's pneumococcus etc. the number of organisms ingested by a leucocyte is relatively small, but that the same serum can be quite freely diluted before phagocytosis ceases. We have found in human beings who were supposedly in good health that a good phagocytic power in concentrated serum may diminish on the one hand in proportion to the degree of dilution, while on the other rapid exhaustion may take place. Under such circumstances we can hardly find it warrantable to conclude that the opsonic content in the two mixtures is identical.

But apart from these considerations there are other reasons which render the determination of the opsonic content according to Wright's method inadvisable for general work. With the

use of concentrated serum it may thus be absolutely impossible to count the number of staphylococci in a given cell. With normal blood or in cases where the phagocytic effect is diminished this difficulty is not so great, but in infections in which the opsonic power is increased enumeration is out of the question. The same holds good when working with the blood serum of certain animals, such as the pig. The number of organisms which are here taken up in concentrated serum is most remarkable; the cells are filled to bursting and accurate counting is impossible. With other organisms there are other difficulties. In the case of the typhoid bacillus, the colon bacillus, the bacillus of Asiatic cholera, and Metchnikoff's bacillus spherulation of the organisms and loss of staining power commonly occur and frequently preclude all possibility of obtaining a satisfactory result by counting. The agglutinating effect of certain sera upon the corresponding organisms is a further drawback to the method, which presupposes that the emulsion of organisms to be added to the blood serum should be uniform and that no agglutinative effect should precede phagocytosis. This difficulty is of special importance when dealing with the tubercle bacillus, and has not yet been satisfactorily overcome.[2] In the case of the anthrax bacillus—and the same applies to Bacillus subtilis—enumeration is for obvious reasons inapplicable and may indeed be impossible.

Led by these considerations, among others, we abandoned the idea of determining the opsonic content of the blood according to the Leishman-Wright method, and have devised the following method, which we believe is more satisfactory and free from the objections which attach to the older procedure.

Method.—By means of a suitably bent and drawn-out glass tube about one half a cubic centimeter of blood, which is readily obtained by the usual puncture of the finger or preferably the ear, is transferred, in portions, to a small tube and mixed with a large excess of a 1.2 per cent. solution of sodium chloride and 0.1

[2] With the method advocated by Wright we could not obtain a satisfactory emulsion of tubercle bacilli. Through the kindness of Dr. E. R. Baldwin of the Saranac Research Laboratory for the study of tuberculosis we were furnished with a supply of bacilli which had been extracted with chloroform. With these much better results were obtained, the bacilli being rubbed up in an agate mortar with 1:1000 salt solution and then centrifugalized. We take pleasure in acknowledging the courtesy of both Dr. Trudeau and Dr. Baldwin.

per cent. of ammonium oxalate. The tube is filled with the same solution and blood and diluent are well mixed and centrifugalized. When the corpuscles are well packed down the supernatant fluid is drawn off with a small pipette and replaced with 1.2 per cent. salt solution, containing no oxalate. The tube is repeatedly inverted and the corpuscles again thrown down by centrifugalization. The washing is repeated once more and the fluid withdrawn down to the corpuscles. The leucocytes which have been thus washed are entirely inactive. In our experiments they were always obtained from supposedly normal individuals and not kept longer than two or three hours, though they may be serviceable for a longer time. Wright and Douglas state that in their experience there was no indication of a variation of the phagocytic power within the space of a few hours, while after the lapse of three days it had declined to less than one half or one third of that of the freshly drawn blood.

The specimen of blood in which the opsonic content is to be determined (about one half cubic centimeter) is collected in a similar manner, but without the use of a diluent. It is placed in a small *dry* tube and allowed to clot, after which the clot is carefully separated from the walls and packed down by centrifugalization. A large drop of the serum which separates out is placed upon a slide and from this various dilutions are made with 1.2 per cent. salt solution. With human blood we usually prepare a 1:20, a 1:30 and a 1:40 dilution, while with the blood of animals a 1:10 and a 1:20 specimen is made. The dilutions are conveniently obtained by the aid of the Thoma-Zeiss pipette used in counting white blood corpuscles. For routine work we use a volume of diluted serum corresponding to eighteen divisions of the pipette. In the case of the 1:30 dilution we thus take twelve divisions of the 1:20 specimen and six of salt solution and for the 1:40 nine of the 1:20 and nine of the salt solution. The resultant preparations are then inoculated with a small amount of the organism under consideration; this is taken from an agar culture and gently rubbed against the tube so that a slight milky turbidity results. If an organism is used which does not readily yield an emulsion in this way, an emulsion must be separately prepared in normal salt solution, or as in the case of the tubercle bacillus in a 1:1000 solution of salt and a small volume of this emulsion is added, due allowance being made for the degree of dilution.[3] Each tube finally receives a constant volume of corpuscles which equals one half that of the diluted serum. The specimens are gently agitated and placed in the incubator at 37°—40° C. for thirty minutes, when large drops of the fluid are placed on slides, spread in the usual manner, (rather thickly) and allowed to dry in the air. It is advisable that in spreading the second slide be merely kept in contact with the blood, without really touching the lower slide; otherwise it may happen that most of the leucocytes, containing organisms, are carried to one end, while only the empty cells are found in the intervening space. When dry the smears are fixed by gently warming them over a flame, when they are stained with an aqueous solution of methylene blue. This has the advantage that in the relatively thick specimens the red cells are not colored and the leucocytes accordingly are more readily found. The use of polychrome dyes is in our experience not at all advantageous.[4] The prepara-

[3] For routine work we used equal parts of a 1:10 dilution of serum and of the tubercle emulsion, thus obtaining a 1:20 dilution.

[4] With the tubercle bacillus Gabbett's method was used.

tions are finally allowed to dry, when the percentage number of phagocyting cells is determined by counting fifty cells which are taken in sequence. When phagocytosis is slight and doubt arises whether in a given cell an organism lies in or upon a cell it is advisable to go over a larger number of cells. With the higher dilution and slight phagocytosis a certain amount of practice is necessary before constant results can be obtained, and even then the personal factor will enter into consideration; this, however, is less disturbing than with the Leishman-Wright method. The results are expressed as in the following example:

1:20	100% +
1:30	92% +
1:40	64% +

Incidentally note is taken in a general way whether many organisms or only a small number are present in the cells.

A problem that gave us much consideration was the selection of the organism for inoculation. As this involves the question of the specificity of the opsonins we shall deal with it under a separate heading. At this place it will suffice to state that for routine work Staphylococcus citreus was employed.

One point upon which we wish to dwell in this connection and which does not seem to have been noticed by previous observers is the necessity of using a time limit during which the tubes are kept in the incubator. In our experiments this was uniformly thirty minutes. If a longer time is allowed the results may be totally different. Generally speaking the number of phagocyting leucocytes increases with prolonged exposure. In one instance the values with the 1:20 dilution were thus 50 after thirty minutes and 80 after two hours.

OCCURRENCE OF OPSONINS IN VERTEBRATES.

Hektoen and Rüdiger[5] have shown that among mammals the opsonins of one species will sensitize bacteria for phagocytosis by the leucocytes of a different species. The animals examined were the guinea pig, rabbit, dog, goat, white rat, and horse. To this list we can add the cat, the sheep, the calf and pig. The serum of all these animals was found to sensitize Staphylococcus citreus for phagocytosis by human leucocytes. We have also found that the same holds good for the serum of the chicken, the terrapin, the frog, and the fish—in other words for all the large classes of vertebrates. The amount of opsonin present in the serum of the various animals is variable, however, as determined by the degree of dilution, and seems to be lower as we descend in the scale of animal life (Table I). The degree of phagocytosis, viz. the number of organisms pro leucocyte, is variable, moreover, not only among the representatives of different species, but also

[5] Hektoen and Rüdiger, *Journ. of Infect. Dis.*, 1905, ii, 128.

among individuals (Table II). To a certain extent these differences may depend upon conditions of nutrition and the process of digestion (*vide infra*), but we feel certain that there are still other factors which are of moment in this connection.

TABLE I.

SHOWING THE OPSONIC VALUES OF ANIMALS OF THE DIFFERENT CLASSES OF VERTEBRATES.

	1:1	1:10	1:20	1:30
Fish	active	—	—	—
Frog	active	—	—	—
Turtle [6]	1:5=52	40	—	—
Chicken	56	0	0	0
Guinea pig		20	12	0
Rabbit		22	10	0
Calf		36	12	0
Sheep		16	0	0
Pig		96	14	0
Dog		32	20	0

TABLE II.

SHOWING THE VARIABILITY OF THE OPSONIC VALUE IN DIFFERENT ANIMALS OF THE SAME SPECIES (ON FIRST EXAMINATION).

		1:1	1:10	1:20	1:30
Rabbits	(122)		—	26	20
	(91)		—	2	0
	(123)		16	0	0
	(131)		2	0	0
	(132)		40	40	28
	(145)		12	0	0
	(162)		72	48	—
	(171)		4	0	0
	(188)		8	0	0
Dogs	(182)		40	28	—
	(214)		24	2	0
	(228)		4	0	0

[6] With the undiluted serum of the turtle there occurs a most extensive agglutination of cocci, with hæmolysis of the red cells and agglutination of the stromata. Under the circumstances it was impossible to obtain any adequate idea of the extent of phagocytosis. On diluting the serum there was still marked agglutination of the cocci, but there was no evidence of hæmolysis.

		1:1	1:10	1:20	1:30
Pigs	(89)	96	14	0	0
	(108)		48	16	4
	(149)	100	8	0	0
	(202)	96	—	—	—
	(240)	96	—	10	—

Rabbit 91 for example is a large buck in excellent condition, with an initial phagocytic value of 2 with a 1:20 dilution, while Rabbit 132 is a much smaller animal and exhibits a phagocytic value of 40 with the same dilution. Dog 228 on the other hand was received in very poor condition and had a phagocytic value of 4 as compared with two other dogs who showed 40 and 50 respectively with a dilution of 1:10, and were much better nourished.

THE OPSONIC CONTENT IN HEALTHY HUMAN ADULTS.

As in the case of individual animals of a given species, so also does the opsonic content of the blood vary in man. But human blood generally speaking contains rather more opsonins than the blood of the lower animals which we have examined.

TABLE III.

TABLE SHOWING NORMAL VARIATIONS OF THE OPSONINS AS ILLUSTRATED BY THE FINDINGS IN TWENTY NORMAL INDIVIDUALS.

	1:20	1:30	1:40
Dr. B.	16	2	0
Dr. D.	44	28	12
Dr. K.	44	24	12
Dr. S.	20	16	4
Mrs. S.	12	8	0
Dr. B.	44	24	18
Dr. L.	56	32	20
M. M.	72	42	12
Dr. E.	44	20	8
Dr. C.	48	28	12
Dr. J.	56	20	16
Dr. F.	18	10	6
Dr. M.	42	28	10
C.	12	6	3
Dr. B.	54	24	8
H. B.	12	4	0
Dr. R.	68	36	24
Dr. T.	24	8	4
Dr. S.	20	16	12
Dr. M.	38	12	2

The values obtained in a series of twenty normal individuals are given in Table III. From this table it will be seen that with a dilution of 1:20 there is phagocytosis in all, the average being 37.2, with 72 as maximal and 12 as minimal values. With a dilution of 1:30 there is also phagocytosis in all, but already diminished in extent, the average being 19.4. With a dilution of 1:40 (average, 9.2) there is a certain percentage of negative results, which increases with the higher dilution of 1:50. Beyond this it is exceptional to find phagocyting leucocytes.

The figures in the table show that the opsonic content of normal blood fluctuates much more than the determination of the opsonic index according to Wright's method would lead one to believe.

In the following apparently normal individuals high values were found.

	1:20	1:30	1:40	1:50
Dr. K.	100	100	100	63
Dr. J.	98	92	48	36
Dr. E.	88	40	40	—
Dr. A.	84	52	20	—
Dr. L.	—	82	18	4

Dr. K. was an individual who was rather a large eater; hardy and vigorous, with no sign of any infection.

Dr. J. was a person of fairly large build, and well nourished.

Dr. E. was a female physician of light build; a very active person.

Dr. A. is of small stature and does not make the impression of unusual vigor. This value was obtained an hour after breakfast.

Dr. L. is a stout man, who is exceedingly prone to pyogenic infections. At the time of examination he was healthy and well. The finding in his case is certainly opposed to Wright's idea that infection takes place because of a low opsonic content.

Our "normal" series includes a few individuals with very low values, which were not included in the table above, as the persons did not make the impression of being in normal health. Dr. N. thus gave 4 for 1:20 and 0 for 1:30 and 1:40 and Dr. B. 6 and 0 respectively. There was no evidence of any actual illness in either, but both looked pale and were rather thin.

All the individuals named in the table were in good health at the time of examination and would hence according to Wright and Bulloch[7] have shown practically the same index, viz. one varying between 0.8 and 1.2. Supposing this to have been the case it is difficult to understand why in one individual the

[7] Bulloch, W., *Lancet*, 1905, ii, 1605.

serum could be diluted fifty times and still retain a certain degree of activity, while in another individual the limit of opsonic power was practically at 1:30, unless we assume, as we do, that the amount of opsonins in the first case was materially greater than in the second.

In a general way the phagocytic power diminishes in proportion to the degree of dilution, but it is noteworthy that in some individuals it is more rapidly exhausted than in others. Under pathological conditions we meet with the same phenomenon, and not infrequently to an accentuated degree.

While the normal range of phagocytic power with a dilution of 1:20 lies between 12 and 72, remarkable exceptions are at times encountered, as in the case of Drs. K., A., and J. and Miss E. It will be observed that in Dr. K. phagocytosis was still active with a dilution of 1:100, being well maintained from 1:20 down. This same good maintenance is seen in Dr. J. and Miss E. We are not prepared to explain these remarkable exceptions. Such findings in our experience are obtained essentially under pathological conditions, but in the instances mentioned there was no indication of the existence of an infection.[8]

The individual variations may in part be due to differences in the state of nutrition, but are not necessarily proportionate to the body weight (Table III). As will be shown in the following section the opsonic content is to a certain extent dependent upon the process of digestion, but in the above series the individual differences cannot be accounted for upon this basis alone, as the examinations in most cases were made in the afternoon about one or two hours after the midday meal.

The values fluctuate to a certain extent from day to day, but remain within the limits indicated.

We repeat at this place that our results have reference to a time limit of thirty minutes in the incubator and that higher values are obtained if this is materially exceeded.

INFLUENCE OF DIGESTION UPON THE OPSONIC CONTENT.

In some individuals a marked influence of the process of diges-

[8] These cases have been separately placed in the above table.

tion upon the opsonic content of the blood can be readily established.

The initial opsonic value of K. at 3 P.M. with a dilution of 1:40 was 100. The same individual after fasting through the night, at 7.30 A.M., showed only 8 per cent. of phagocytosis with a dilution of 1:30. The results at 2 P.M., one hour after a hearty dinner, were the following:

1:30=98 1:40=96 1:50=96

Two hours later the corresponding figures were:

1:30=86 1:40=74 1:50=26

The rapid exhaustion as compared with the results obtained one hour after dinner, is here strikingly shown. The same is seen early in the morning. In the given experiment a 1:20 determination was not made at that hour, but on another occasion the value was 82, and fell to 2 with a dilution of 1:40.

At 6.50 P.M. supper was had and one hour later higher values are again found, which are well maintained with the higher dilutions, viz.:

1:30=98 1:40=98 1:50=98

The figures given in conection with this experiment should not be regarded as averages, however, as they were obtained in the case of Dr. K., who showed an exceptionally high phagocytic value.

The same general result was obtained in the case of a rabbit.

The initial phagocytic value was 4 per cent. for 1:30, and negative for 1:40. After fasting absolutely for 43 hours there was no phagocytosis whatever with 1:30; with 1:20 only 2 per cent. of the cells were positive, and with 1:10 only 20 per cent. The animal was then well fed and about one hour later gave the following values:

1:30=28 1:40=22 1:50=6

Dr. Amberg has made a number of opsonic determinations according to our method in breast-fed babies in the obstetrical department of the Johns Hopkins Hospital. He will report the details of his results at another place, but has kindly placed his general conclusions at our disposal. From these it appears that in nursing infants the opsonic content is distinctly higher than in the average normal adult, and that dilution is well borne up to 1:100 and in some instances even higher.

Baby No. 8	1:20	84
	1:40	70
	1:80	48
	1:100	22
Baby No. 9	1:25	80
	1:50	64
	1:75	30
	1:100	20

It seems to us that these high values are to a certain extent at least referable to the fact that the nursing infant is practically in a condition of continuous digestion.

But even though a distinct influence upon the opsonic content by the process of digestion is apparent in certain cases this is not manifest in all.

In the case of one of us (S) higher values were thus obtained early in the morning before any food had been taken than two hours after the midday meal. Miss E. similarly showed high values even after fasting absolutely for twenty-one hours, viz.:

1:20 = 92 1:30 = 48

The same individual was placed upon Folin's non-proteid diet for forty-three hours and at the end of this time still showed very active phagocytosis:

1:20 = 88 1:30 = 40

In rabbits also we have found that good feeding *per se* does not increase the opsonic content of the blood beyond the initial values which were noted shortly after the animal had been obtained from dealers. Individual peculiarities exist here, of the causes of which we know nothing as yet.

From a practical standpoint in the study of infections, however, it is well to bear the possible influence of digestion upon the opsonic content in mind. The fact that such a relation may exist throws additional light upon the rationale of "feeding a fever." Moreover, the high figures obtained by us in certain infections thus stand out in even greater contrast, if we bear in mind that in many of these cases the food consumption had been much diminished.

DISTRIBUTION OF THE OPSONINS IN THE ANIMAL BODY.

In order to ascertain what part, if any, the various organs of the body take in the production of opsonins, rabbits were bled by dividing the femoral vessels and thoroughly milking the body. The organs were then removed and ground up with well washed and dried quartz sand. In the case of the liver it was possible to obtain a sufficient quantity of fluid extract in this manner without the addition of any diluent, but in the case of the other organs it was necessary to use a small amount of normal salt solution. Whenever this was done a large amount of the triturated mush was placed in a tube, digested with the salt solution, and the sand and cellular elements then separated out

by centrifugalization. The degree of dilution was in every case as slight as possible, merely enough to procure a few drops of liquid extract. This was then placed in a separate tube, infected as usual with Staphylococcus citreus, treated with washed human corpuscles, incubated and further examined as described. The results were absolutely negative in the case of striated muscle tissue, liver, spleen, lymph glands, kidneys, intestinal mucous membrane and muscular coats. For the examination of the adrenal glands, brain, pancreas, testicles and ovaries sheep were chosen and for the thymus gland the calf. The results were uniformly negative.

Examination of the bone marrow did not yield entirely satisfactory results. The material was obtained from the bones of a cat, stirred up in a small amount of normal salt solution and incubated at 37° C. for one hour and a half. The cellular material was then removed as far as possible by centrifugalization and the liquid portion examined. It was found that phagocytosis had occurred in 12 per cent. of the washed corpuscles. We fear, however, that the positive result in this case may have been due to the presence of blood serum which could not be removed.

Transudates like blood serum also contain opsonins, but the amount is smaller in the former. With a specimen of fresh hydrocele fluid we thus found only six per cent. of phagocyting leucocytes for a dilution of 1:20 and with a sample of ascitic fluid 10 per cent. with the same dilution. Chyle contains rather more, but also less than the blood serum (60 per cent. in one case in concentrated form).

Our results thus tend to show that the opsonins are essentially components of the blood and the inference naturally suggests itself that they may be formed here. The fact that they are increased so frequently in infections which are associated with hyperleucocytosis would seem to point to the leucocytes as their possible source. Our investigations, however, do not support this view. They show conclusively that no constant parellelism exists between the number of leucocytes and the amount of opsonins. This is well illustrated by Fig. 1, which was obtained

in the case of a dog, in which an obstruction of the small intestine had been artificially produced; this experiment is one of a series which one of us (Simon) undertook in association with Dr.

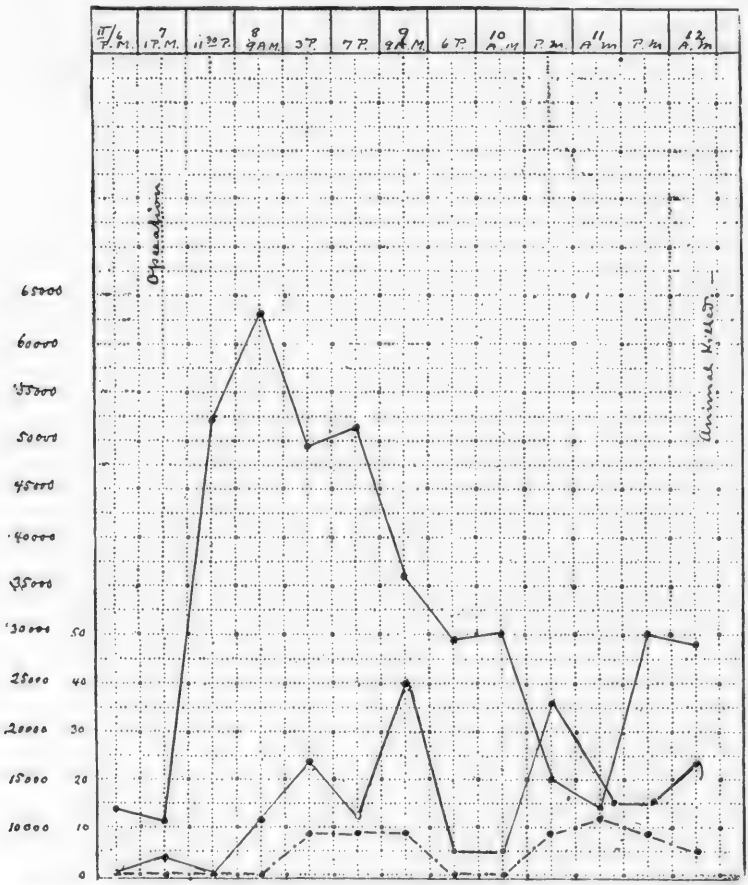

FIG. 1.—Upper solid line=total leucocyte count; lower solid line=opsonins, 1:10 (percentage values); broken line=opsonins, 1:20.

Nelson for the purpose of studying blood changes in intestinal obstruction, a detailed report of which will appear in another place. The animal in this case survived and was subsequently

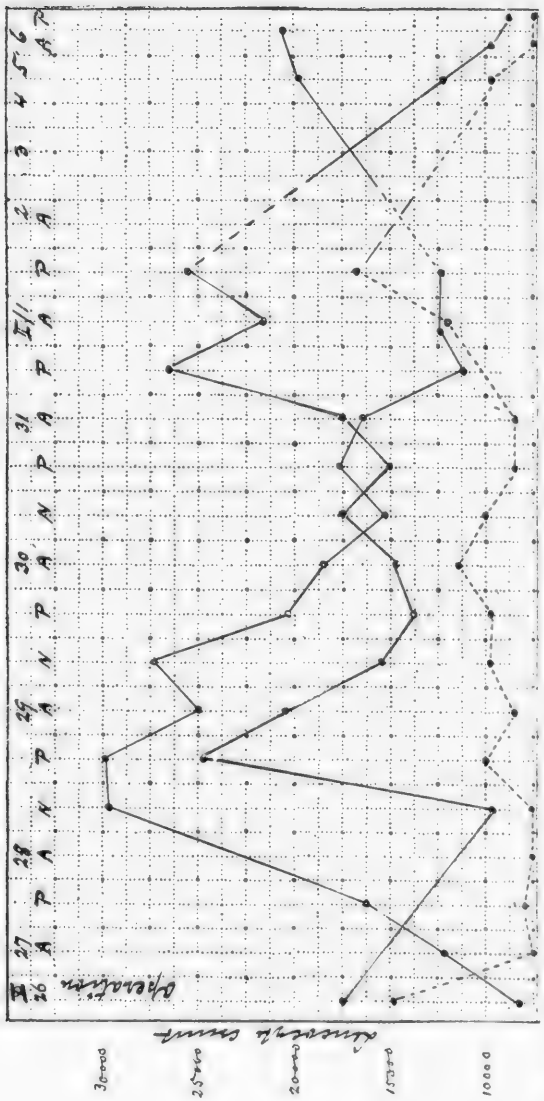

FIG. 2.—Upper solid line=total leucocyte count; lower solid line=opsonins 1:10 (percentage values); broken line=opsonins 1:20.

killed, when it was found that the calibre of the gut had been narrowed sufficiently to allow only the passage of a large sound.

It will be observed that notwithstanding the remarkable increase of the leucocytes, to a point above 60,000, the opsonins both for the 1:10 and the 1:20 dilution gave values which were not at all in excess of what is normal for dogs; for the most part in fact the values were rather lower than those commonly found. The same is seen in Fig. 2, which was obtained from another dog that had undergone a similar operation.

Examination in the case of patients led to the same conclusion. This is well illustrated by the two following observations.

In both cases the symptoms were suggestive of appendicitis. In the one (No. 37) the leucocytes just before operation numbered 23,000; the phagocytosis with a dilution of 1:20 was 18 and negative with 1:30. The appendix in this case was practically normal; there were two patches of induration near the lower end of the ileum and one at the base of the appendix. A definite diagnosis could not be reached; but a history of recent syphilis was considered suggestive. In the second case (No. 68) the leucocytes numbered 23,200—practically the same number as in the first, while the opsonic examination showed 100 per cent. of phagocyting leucocytes even with dilutions of 1:30 and 1:40.

Conversely cases occur where with a normal number of leucocytes the opsonins are very high. This is well shown in the following case:

The patient (No. 137) was a young married woman who was admitted to Dr. Finney's service at the Union Protestant Infirmary with a greatly enlarged spleen and liver. The differential diagnosis rested between Banti's disease and syphilitic cirrhosis. The leucocytes numbered 7500, while phagocytosis with 1:20 was 100 and with 1:30, 96, *i.e.* greatly above the normal.

While the clinical evidence was thus opposed to the assumption of a relationship between leucocytes and opsonins we have made further examinations in this direction, by incubating washed leucocytes for variable lengths of time, suspended in normal salt or in Ringer's solution, and subsequently examining for the occurrence of phagocytosis. The results were invariably negative.

While the above observations render it unlikely that the neutrophilic and amphophilic granulation is concerned in the production of opsonins the rôle of the eosinophiles still remained doubtful. In the case of the incubation experiments just

referred to it was conceivable that the small number of eosinophiles in our tubes were not sufficient to bring the opsonins up to that degree of concentration at which phagocytosis would occur. To throw some light upon this question a rabbit was fed about 1750 trichinæ. A marked eosinophilia resulted, which at first was accompanied by a rise in the opsonins. Subsequently, however, they fell and at the last examination no phagocytosis whatever occurred with a dilution of 1:10.

The lymphocytes, according to our negative findings in the case of the lymph glands, can be excluded as opsonin producers.

In the cerebro-spinal fluid from a case of spina bifida we were much surprised to find no evidence of the presence of opsonins.

The examination of fresh seminal fluid and of milk gave negative results.

In exudates the presence of opsonins is variable; but as the element of infection enters into consideration in their production they will be dealt with under a subsequent heading.

CHEMICAL STUDY OF THE OPSONINS.

Our chemical investigations were rendered particularly difficult owing to the comparative instability of the opsonins; viz. their partial thermolability and the readiness with which various chemical reagents lead to their destruction, or at least inactivation so that they are no longer subject to detection by their specific effect upon the leucocytes. Hektoen and Rüdiger have shown that various salts even in very small amounts are capable of suspending opsonic action when added to the blood serum. They determined this in the case of calcium, barium, strontium and magnesium chloride, potassium sulphate, sodium bicarbonate, trisodium citrate, sodium oxalate, potassium ferro-cyanide and formalin. It was accordingly necessary by means of a series of preliminary experiments to determine what physical processes and what chemical reagents could be utilized without interfering with opsonic action. Then again it was necessary to ascertain, *ceteris paribus*, how long the opsonins will remain active in the blood serum. Wright and Douglas state that the opsonic power

of the blood fluids disappears gradually on standing, even when the serum is kept in a sealed capsule, sheltered from light. Under these conditions they found the opsonic power after five or six days to stand at a little more than half of what it was originally. This observation we are able to confirm. With the serum of the pig and of the rabbit we found a vigorous degree of opsonic activity after a week and even longer, no matter whether the serum was kept in the cold or at the temperature of the laboratory. It was interesting to note that with a fair development of bacteria in the serum, we could obtain good phagocytosis after from six to eight days; leucocytes were then found which had taken up the bacteria which had grown in the serum.

We have stated before that Wright and Douglas could demonstrate a partial thermolability for the opsonins, inasmuch as the number of ingested organisms in heated serum was usually less than in unheated serum. But apart from the diphtheria bacillus and Bacillus xerosis, which were taken up as well in the one as in the other, a complete extinction was not as a rule obtained even with those organisms which Wright designates as especially susceptible to opsonic action. In his first experiment with the typhoid bacillus[9] he thus notes that in the heated specimen there was "everywhere considerable phagocytosis" and the phagocytic index is given as 26.2 (circ.) as compared with 24 (circ.) in the unheated serum. In Experiment II with the same organism, on the other hand, the value was 8.1 in the unheated and only 0.8 in the heated one.

Other observers seem to have taken the complete thermolability of the opsonins for granted. But Dean[10] has recently shown that such an assumption is not warrantable and that Wright's results which suggest this possibility are referable to an inherent inaccuracy of the method, viz. the small amount of serum which is used. He could show that normal horse serum, even after being kept for four hours at a temperature of 60° C., still contains a quantity of opsonins sufficient to prepare large numbers of

[9] Wright and Douglas, *Proc. Royal Soc.* 1904, lxxiii, 137.
[10] Dean, G., *Centbl. f. Bakt.*, Ref., 1905, xxxvii, 349, 449.

cocci for phagocytosis. Similar results were obtained by Smith [11] in the case of typhoid immune sera. In a recent communication Wright [12] attempts to show, on the other hand, that the phagocytic effect of heated sera is merely referable to opsonins which have escaped destruction and that such sera when diluted with 1.2 per cent. salt solution and subsequently heated are inactive.

Behavior on dialysis.—To study the effect of dialysis upon the opsonins small quantities of blood serum (25–50 c.c.) were placed in sacs of animal membrane or of celloidin and dialyzed against running water for from forty-eight to sixty hours. After this time it was found that the remaining fluid showed no opsonic effect whatsoever, but the appearance of the leucocytes suggested that the negative effect might very well be due to the atonicity of the dialyzed fluid. Upon the addition of a corresponding quantity of Ringer's solution to the dialyzed serum active phagocytosis could again be obtained, although to a less extent, so far as the number of phagocyting leucocytes goes, than with the native serum. This diminution in the amount of opsonins upon first consideration suggests that the substances are to a certain extent dialyzable, but our subsequent experiments have placed an entirely different light upon this apparent loss, so that we believe that we are justified in concluding that the opsonins are not dialyzable. The active phagocytosis which was obtained with dialyzed serum plus Ringer's solution shows that the soluble phosphates and carbonates of the blood are not essential to the process. The fact that the same result also was obtained with dialyzed serum plus normal salt solution further shows that the soluble calcium salts and sulphates of the serum were likewise not of moment. The suggestion that the decalcification of the blood *per se*, by means of the ammonium oxalate or sodium citrate used in washing the leucocytes, was essentially responsible for the non-occurrence of phagocytosis we had previously invalidated by showing that the addition of Ringer's solution to washed corpuscles, without serum, does not restore phagocytic action.

[11] Smith, R. G., *Proc. Linnean Soc.*, New South Wales, 1905, ii., 269. Wright and Reid, *Biochemisches Centbl.*, Ref., 1906, iv, 125, 126.

[12] Wright and Reid, *Proc. Royal Soc.*, 1906, lxxvii, Series B., 194.

As the addition of normal potassium chloride solution to the dialyzed serum was found to answer the same purpose as the sodium chloride it appears likely that the function of the chloride is in a manner secondary, and merely serves the purpose of maintaining the necessary osmotic tension of the leucocytes. As we have shown, its temporary abstraction during dialysis does not interfere with the subsequent action of the opsonins when the necessary quantity is restored.

While our experiments with dialysis show that the opsonins must be sought among the non-dialyzable components of the blood serum, our experience with the soluble extractives of the various organs suggests that they are specific constituents of the serum. It would hardly seem likely that lecithins, cholesterins, soaps etc. are of moment.

Bearing in mind the close relationship which seems to exist between certain antibodies and the blood albumins experiments in this direction seemed to be indicated, and were rendered possible by our discovery that ammonium sulphate, even when added in substance to saturation, does not destroy the opsonins, although their specific effect can only become manifest again after the excess of salt has been removed by dialysis.

In a series of experiments the albumins of fifty cubic centimeters of serum were thus collectedly precipitated by salting at 40° C.: the precipitate was dissolved in as small a bulk of water as possible and dialyzed against running water for at least forty-eight hours. The contents of the dialyzer were then concentrated in the vacuum to about one half the original volume, diluted with double the amount of 1.2 per cent. salt solution and examined in the usual manner. Active phagocytosis occurred, although the number of phagocyting cells was materially smaller than with the original serum.

The filtrate, after salting out the albumins, as just described, was likewise dialyzed against running water, concentrated in the vacuum to a small volume and treated with salt solution and examined as above. There was no phagocytosis whatever. After having thus demonstrated that the opsonins can be isolated in association with the blood albumins, the albumins were

fractioned in the usual manner. The globulin fraction was isolated by half saturation with ammonium sulphate, the precipitate dissolved in a small amount of water and dialyzed. In the water-insoluble portion which separates out (euglobulins) opsonins could then be demonstrated, if a saturated solution in 1.2 per cent. salt solution was prepared and examined in the usual manner, while the pseudoglobulin fraction after concentration in the vacuum, as also the serum albumin fraction, were entirely inactive.

The opsonic content of the euglobulin fraction was less than that of the original serum, but still quite considerable. In one experiment where the examination with the native serum had shown 100 per cent. of phagocyting leucocytes, the concentrated euglobulin solution showed 74 per cent. of the cells still quite active. On diluting, however, to the extent of only 1:10 the result was entirely negative.

These experiments were repeated a number of times with the serum of pigs, calves, sheep, and also with ascitic fluid, and in all cases with the technique described the opsonic effect was obtained with the euglobulin fraction and with this only. The conclusion hence suggested itself that the opsonins, even though themselves not necessarily of the nature of globulins, were nevertheless intimately associated with them as in the case of the various antibodies as shown by Pick.[13] But whereas Pick could demonstrate that diphtheria antitoxin, for example, was quantitatively precipitated from goat's serum in the globulin fraction, the material loss which we met with made us hesitate to regard this conclusion as altogether valid. It seemed not at all impossible that the opsonins, like ferments, were carried down mechanically and in part lost during the physical manipulations (filtration, dialysis), viz. deposited upon the walls of the dialyzer etc. While we have not been able to prove conclusively that this actually occurs, certain experiments which were undertaken to elucidate this point certainly are very suggestive. To this end the quantity of opsonin was first estimated in the native serum. Small tubes were then charged with small quantities of serum

[13] Pick, E., *Hofmeister's Beiträge*, 1901, i., 351.

together with various substances which in themselves were not thought to exert a destructive effect upon the opsonins, such as coagulated egg albumin which had been dried and finely powdered, wheaten flour, diatomaceous earth, precipitated chalk, filter paper fibre, vegetable charcoal, carmin and platinum black. After having shaken the tubes thoroughly for one half hour the suspended material was thrown down by centrifugalization and the serum examined in concentrated form. The figures representing phagocytosis are given in Table IV.

TABLE IV.

Native serum	48
Carmin	40
Platinum black	40
Diatomaceous earth	36
Precipitated chalk	14
Filter paper	4
Albumen	0
Charcoal	0
Flour	0

These experiments show conclusively that the opsonins can be in part or entirely removed from blood serum by various substances in a manner quite analogous to what we see in the case of various ferments. That they are not destroyed can be shown in the case of carmin and charcoal, for example, by washing the material in 1.2 per cent. salt solution and then exposing it to the action of washed leucocytes, when numerous cells are seen which have taken up the fine granules.

A perfectly analogous result was obtained with cream of milk. Cream was thoroughly shaken with serum for half an hour, separated by centrifugalization and washed with 1.2 per cent. salt solution. With a particle of the resultant butter a fine suspension was prepared in salt solution and incubated for fifteen minutes with washed corpuscles, A drop of this mixture was mixed with a drop of a one per cent. solution of osmic acid and examined, when it was seen that a certain degree of phagocytosis had occurred.

It might of course be argued that the phagocytosis which was thus obtained with carmin, charcoal and fat globules was not

dependent upon the absorption of opsonins, but was of the character of spontaneous phagocytosis. This, however, is negatived by the fact that in all experiments a 1.2 per cent. solution of salt was used, which according to Wright eliminates spontaneous phagocytosis. Upon the basis of these experiments it seems not inconceivable that the cause for our finding of the opsonins in the euglobulin fraction of the blood serum may be sought in the fact that these are the only albumins which in the nature of our technique are precipitated on dialysis and would hence carry the opsonins with them. This possibility must be borne in mind in future research. On the other hand our loss of opsonins during our manipulation might be referred to the time element which enters into consideration and would simply represent that portion of opsonin which would become inactive in the native serum upon standing for a corresponding length of time. To enter into this question further did not seem desirable at the present time.

The absorption experiments tend to throw some light upon the nature of the sensitizing of bacteria and the differing behavior of various organisms in reference to the extent of phagocytic action. Similar conditions obtain with various ferments, such as pepsin, which is readily absorbed by animal charcoal, diatomaceous earth, coagulated serum and egg albumin, etc., while bread, flour, lecithin and cholesterin are less active.

SPECIFICITY OF THE OPSONINS.

The question regarding the specificity of the opsonins is an exceedingly important one. From the standpoint of technique in the investigation of the opsonic content it would simplify matters very much, if it could be shown that the opsonins are not specific, while such a demonstration would also modify present conception regarding the mode of action of bacterial vaccines. As Bulloch[14] states, "this question has hitherto not been touched in the various memoirs," although Wright manifestly assumes that it has been settled in the affirmative. Bulloch comes to the same conclusion, viz. that a high degree of specificity

[14] Bulloch, W., *Lancet*, 1905, ii, 1603.

exists. His inference is based upon two classes of experiments. "In the first the opsonic power of a serum was tested against both Staphylococcus albus and the tubercle bacillus. The serum was then mixed with one or other of these microbes and after a sojourn in the incubator the mixture was subjected to the prolonged action of the centrifuge, whereby the microbe was thrown down as a deposit; the supernatant fluid was deprived of its opsonins for the particular microbe with which it had been in contact, while it 'largely' [sic] retains its opsonins for the microbe with which it has not been digested." Unfortunately Bulloch gives no details of his experiments, from which the reader can form an estimate of what is meant by the statement that the serum "largely retains its opsonin for the microbe with which it has not been digested." We must confess that we see no cogent reason why, assuming the high specificity of the opsonins, the opsonin in the experiments mentioned should have been only "largely" retained.

Our own experiments were conducted in an analogous manner and led to results which are diametrically opposed to Bulloch's conclusion, so far as Staphylococcus citreus, on the one hand, and the typhoid bacillus and colon bacillus on the other are concerned. In the end, as usual, we determined the number of phagocyting leucocytes. In one of these experiments the opsonic power of the native serum (of the pig) was 100 both for the colon bacillus and Staphylococcus citreus. A considerable portion of an agar culture of Staphylococcus citreus was then emulsified in about three cubic centimeters of serum, the mixture stirred for at least fifteen minutes and the organisms thrown down by prolonged centrifugalization. Specimens of the supernatant fluid were then reinfected with staphylococci, on the one hand, and colon bacilli, on the other, and re-examined. It was found that the opsonic power for the staphylococcus had fallen to 20 and for the colon bacillus to 14.

To meet the possible objection that the 20 per cent. for the staphylococcus may have been referable to the presence of sensitized cocci which had not been thrown down by centrifugation the experiment was repeated, but the centrifugalized

serum treated with an abundant supply of washed corpuscles and incubated for fifteen minutes so as to remove the sensitized cocci as far as possible by phagocytosis. The corpuscles were then thrown down and the supernatant fluid examined as before; in this case the result was 16 for the staphylococcus and 20 for the colon bacillus.

These experiments were repeated again and again and variously modified. In one instance for example the serum was incubated with a large supply of colon bacilli and kept in the incubator for twenty hours. At the end of that time it was tested as described; six per cent. of the leucocytes showed phagocytosis for the colon bacillus and four per cent. for the staphylococcus. There was accordingly no indication whatsoever of any specificity of the opsonins; exhaustion for both organisms proceeded *pari passu* in all our experiments.

The same conclusion is suggested by our observation that the opsonic coefficient of extinction is practically the same for different organisms, and irrespective of the initial intensity of phagocytosis, as shown in Table V.

TABLE V.

SHOWING THAT THE OPSONIC COEFFICIENT OF EXTINCTION IS THE SAME FOR DIFFERENT ORGANISMS (PIG SERUM).

	1:10	1:20	1:30	1:40
Staphylococcus citreus	48	16	4	0
Spirillum Metchnikovi	24	20	8	0
Bacillus coli	44	24	16	0
Bacillus typhosus	52	24	4	0
Bacillus Pfeifferi	22	16	16	0
Bacillus anthracis	36	24	8	0

In a second class of experiments Bulloch reports that the serum of human beings was tested repeatedly both against the tubercle bacillus and against the staphylococcus. He finds that injections of tuberculin produced an increase in the "tubercular" opsonins, while leaving the quantity of "staphylococcus" opsonins unaltered and *vice versa*. It seems to us that experiments of this character are not well suited to settle the question under consideration. In such cases we are essentially

dealing with sera in which the entire immunizing mechanism of the body is without doubt thrown into activity and in which other factors besides the opsonins may be affected. We believe Wright and his collaborators have conclusively established the existence of opsonic substances in the blood serum, but we are not prepared at the present status of our knowledge of the subject to admit that no other factors enter into consideration in connection with the process of phagocytosis. It is quite conceivable to our minds that as the result of infection, viz. immunization, substances may be produced either *de novo*, or increased in amount, if pre-existent, which may so influence a given organism that under the subsequent action of a common non-specific opsonin it is taken up in larger or smaller numbers, as the case may be.

This possibility that a second substance besides the opsonins may determine the number of organisms phagocyted, also exists in the case of normal blood serum. It is strongly suggested by the fact that in some individuals a very small number of cocci only is taken up with a dilution of 1:20, for example, so small indeed that we would expect no phagocytosis at all with 1:30 and 1:40, reasoning by what we see in most cases; this dilution may nevertheless be borne without extinction of the phagocytic power. We have repeatedly observed that with a dilution of 1:20 not more than four or five staphylococci are taken up by the phagocyting cells, and that with a dilution of 1:30 we still find two or three and with 1:40 perhaps the same number of organisms. On the other hand we have found that with a fairly large number of cocci pro cell, with a dilution of 1:20, there may be practically no phagocytosis with 1:40. It would seem as though the quantity of opsonins in these cases was fairly uniform, but that there must be another factor which is variable.

This assumption receives a certain amount of support from the following observation:

To eliminate opsonic action blood serum of a pig was diluted one hundred times, infected with cocci and incubated for one half hour. Blood serum from an individual, whose phagocytic value with a 1:20 dilution was 50, was then diluted

with the diluted pig serum in the proportion of 1:20 and an increase in its phagocytic value to 80 noted. The diluted pig serum *per se* when treated with washed corpuscles showed no phagocytosis and a control specimen with 1.2 per cent. salt solution likewise was negative.

Even more suggestive is the following:

One of our rabbits has shown a remarkably low opsonic content, such that no phagocytosis was ever obtained with a 1:20 dilution and only rarely with 1:10 and then only to a slight degree, not exceeding 8. Pig's serum, which with a dilution of 1:20 gave a phagocytic value of 10, was diluted 100 times, infected and incubated for one half hour. The rabbit's serum by itself on this occasion showed 1:10=4 and 1:20=0, but when diluted 1:20 with the diluted pig serum phagocytosis was 47 and the cells contained a fair number of cocci. Pig serum was chosen for these experiments because the phagocytic value for staphylococci is exceptionally great, when used in concentrated form, although it is rapidly lost on dilution.

In order to invalidate the argument that the preceding incubation, previous to the 1:20 dilution, might be responsible for the increased phagocytosis salt solution was substituted for the 1:100 dilution of serum and this incubated with cocci for half an hour. The result showed no difference between the original 1:20 dilution of the serum and the subsequent 1:20 dilution with the incubated cocci; the phagocytic value in both was exactly alike, viz. 4.

It might further be argued that in some manner the opsonins in the 1:100 solution, even though they be insufficient in this dilution to bring about phagocytosis by themselves, become united with the opsonins of the serum, which is about to be diluted 1:20, and that the joint effect then becomes manifest. Upon a mathematical basis *per se* this would appear most unlikely; we have shown, however, by experiments of the following nature that this explanation is not valid. To this end the blood serum of one and the same person was used, instead of two different individuals. A 1:20 dilution was prepared as usual, as also a 1:100 dilution. The latter was incubated for half an hour after infection with cocci and a new dilution of 1:20 prepared using the hundredfold dilution in lieu of the salt solution as usual. The result shows conclusively that the 1:100 dilution is not capable of raising the phagocytic value of the same individual to a higher level than is obtained primarily. The values in a given case were 38 for the primary 1:20 dilution and 36 for the second dilution.

Experiments of this order have incidentallv shown the interesting fact that on diluting the blood of one person with that of

another, or of one animal with that of another a higher phagocytic value *may* be obtained, but is not necessarily obtained, than in the case of either alone, when diluted with salt solution. This peculiar behavior we are not able to explain at the present time. The problem, however, is reserved for further investigation.

But even admitting the previously mentioned observations of Bulloch and Wright to consideration for the sake of argument, it appears to us that equally cogent reasons can be advanced to prove the non-specificity of the opsonins along similar lines. It is thus difficult to understand why the opsonins for Staphylococcus citreus should be increased in cases in which no evidence of a staphylococcus infection exists whatsoever. In a case of syphilitic cirrhosis of the liver, the phagocytosis for the staphylococcus was thus 100 with a dilution of 1:20 and 96 for 1:30. In a case of extensive miliary carcinomatosis the same values practically were found. The same was found in a case of pernicious anæmia. In the trichinous rabbit similarly there is certainly no ground for belief that an associated staphylococcus infection existed. In a rabbit which had died as a result of a streptococcus infection and in which cultures from the various organs and from the heart blood revealed this organism only, the opsonins for Staphylococcus citreus were enormously increased, viz. at 92 for a dilution of 1:10. In a case of melanotic sarcoma further, in which an extensive excision had been done and in which the opsonic power was quite low, a marked rise, as tested by staphylococci, was observed as the result of Coley's mixed toxin injections, viz. the toxins of Streptococcus erysipelatosus and of Bacillus prodigiosus.

Our findings in cases of appendicitis also may be cited in this connection. Maximal values are here commonly observed and in accordance with the usual bacteriological findings can hardly be referred to a mixed infection of colon bacilli, streptococci and staphylococci.

We have further determined the opsonic content of the blood of tubercular individuals, with our method of dilution, both against Staphylococcus citreus and the tubercle bacillus, and have found the coefficient of extinction the same for both organisms.

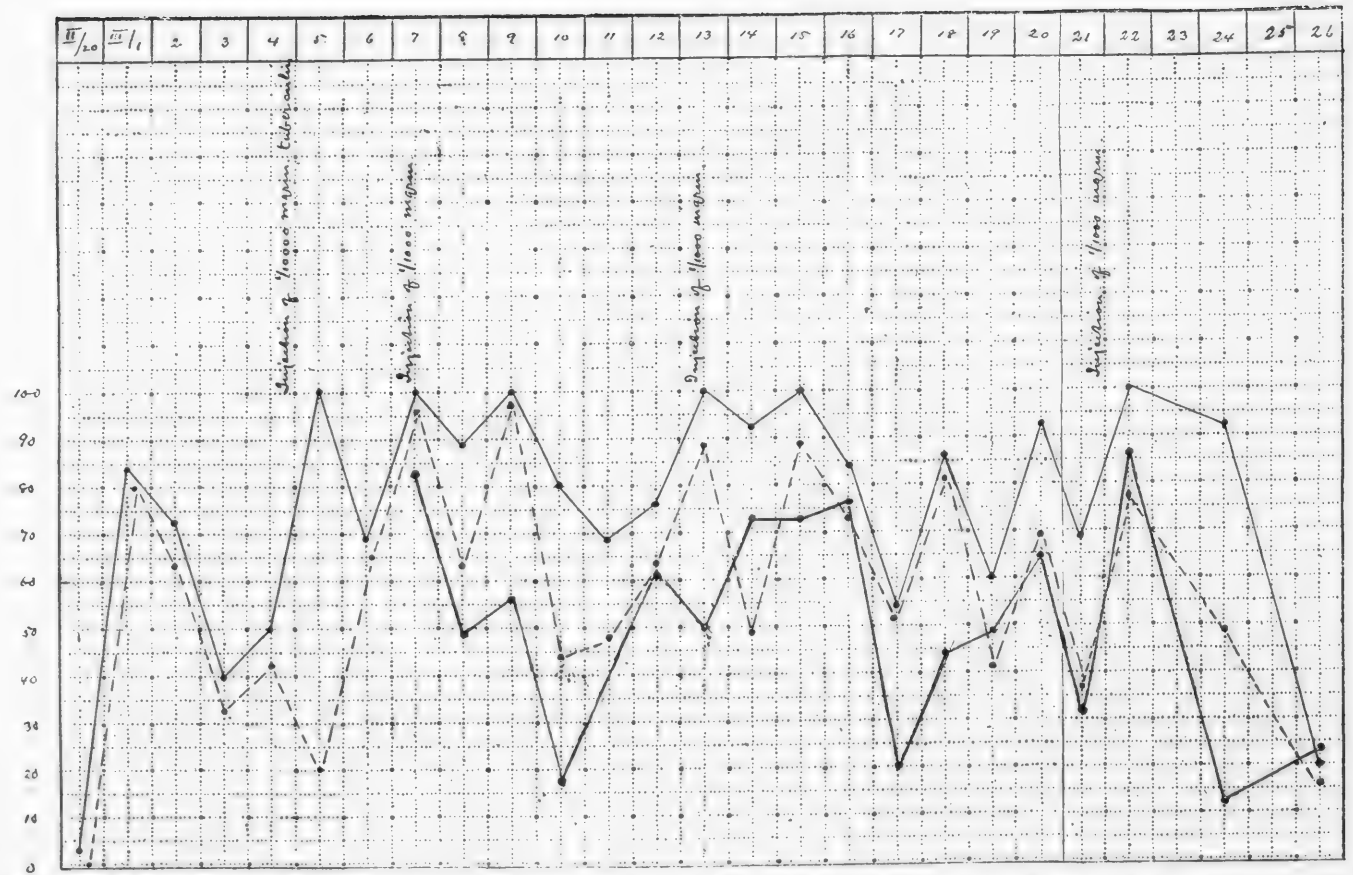

FIG. 3.—Upper solid line represents serum diluted 1:20 (staphylococcus); broken line, a dilution of 1:30 (staphylococcus); lower solid line, 1:20 (tubercle bacillus.)

In one instance of renal tuberculosis for example the value for the staphylococcus with a dilution of 1:20 was 3 and for the tubercle bacillus 2, while with 1:30 there was no phagocytosis with either organism.

Fig. 3, moreover, which illustrates the effect of tuberculin injections upon the opsonic curve, as tested simultaneously with the tubercle bacillus and the staphylococcus, shows that the two run essentially a parallel course and rise and fall together. The minor differences are unquestionably due to the difference between the organisms and their initial variability, corresponding to what we have demonstrated in the case of other organisms (*vide supra*). It is noteworthy also that, although in this case there was no evidence (by culture) of any staphylococcus infection, the staphylococcus curve was nevertheless unusually high and manifestly influenced by the tuberculin injections.

The good phagocytic values which were obtained in cold blooded animals, finally, in which staphylococcus infections hardly occur, would similarly argue against the specificity of the opsonins.

Summing up these various observations, we believe we are justified in concluding that specificity of the opsonins does not exist. Our experiments suggest the possibility that the opsonins may be a constant quantity and that the number of organisms which is taken up by a cell is influenced by a second factor which may be variable.

THE INFLUENZA BACILLUS IN INFLAMMATIONS OF THE RESPIRATORY TRACT IN INFANTS.*

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The object of the following study is twofold: to determine the presence of influenza-like bacilli in the throat and bronchial mucus of children with and without pulmonary inflammations, and to arrive at a differentiation of these bacilli by means of cultural characters and agglutination reactions.

The material was chosen with a view to including as many different pulmonary conditions as possible. A series of five normal infants, whose history showed no cough since birth, was first examined with negative results. Cases of coryza, bronchitis, broncho-pneumonia, lobar pneumonia, empyæma, rachitis, gastro-intestinal diseases, typhoid fever, diphtheria, tonsillitis, measles, general sepsis following mastoiditis, rheumatism, and cerebro-spinal meningitis, with and without pnuemonia, were next studied. The cases of coryza, bronchitis, and pneumonia were examined within from twenty-four to forty-eight hours after admission, whether the onset had been such as to suggest influenza or not. Only those cases were diagnosed "influenza" which showed the influenza bacillus in the bronchial or nasal secretions. I am indebted to Dr. L. Emmet Holt for the opportunity of studying twenty-two cases of measles at the Foundling Asylum. Of the cases which came to autopsy at the Babies' and Foundling Hospitals blood-agar plates were made from the lungs irrespective of the cause of death.

The method employed was as follows; a sterile swab was rubbed over the posterior pharangeal wall, care being taken to avoid the tongue and the roof of the mouth. In most instances

* This investigation was aided by a grant from the Rockefeller Institute for Medical Research.

this operation causes cough and the raising of some mucus from the bronchi, to which the swab attaches itself. Whenever the mucus was obtained in sufficient quantity, it was washed according to the method of Koch and Kitasato and then spread on blood-agar plates. A similar method was used by Jündell.[1] Rabbit blood was used and mixed with the melted agar at 50° C. When only a very little secretion was obtained, this was spread at once upon the blood-agar plates. The throat cultures were always taken two or two and a half hours after a meal. The plates were examined after seventeen to twenty hours incubation. If no colonies of influenza-like bacilli were found, they were again incubated and re-examined the next day. Only those plates in which the colonies were definitely characteristic and proven by impression preparations to be made up of bacilli having the size, shape, and staining reactions of Bacillus influenzæ, were regarded positive. In the majority of instances subcultures on blood-agar plates served to isolate these bacilli in pure culture, but in some I failed to obtain pure cultivations because of the presence of other organisms, chiefly cocci, in predominant numbers.

CULTURES MADE DURING LIFE.

	POSITIVE		NEGATIVE		Total No. of Infants Examined.
Broncho-pneumonia	16		37		53
Lobar-pneumonia	1		7		8
	With Pneum.	Without Pneum.	With Pneum.	Without Pneum.	Total
Normal baby	0	0	0	5	5
Bronchitis	0	11	0	34	45
Empyæma	3	0	4	0	7
Coryza	0	1	0	2	3
Tonsillitis	0	0	0	3	3
Diphtheria	0	0	0	4	4
General sepsis after mastoiditis	0	0	0	1	1
Cerebro-sp. meningitis	1	0	2	7	10
Rheumatism	0	0	0	1	1
Rachitis	0	0	0	4	4
Gastro-intestinal diseases	0	0	0	22	22
Typhoid fever	0	0	0	3	3
Measles	3	5	5	13	26
Tuberculosis	6	2	12	4	24

CULTURES MADE AT AUTOPSY.

	POSITIVE		NEGATIVE		TOTAL
Broncho-pneumonia	3		10		13
	With Pneum.	Without Pneum.	With Pneum.	Without Pneum.	Total
Tuberculosis	5	1	0	2	8
Measles	1	0	0	0	1
Entero-colitis	0	1	0	3	4
General (umbilical) sepsis	0	1	0	1	2
Prematurity (atelectasis)	0	1	0	2	3
Marasmus (bronchitis)	0	1	0	0	1

The ages of the children varied from three days to four years, as follows:

Under 3 months old	18
Between 3 and 6 months old	40
" 6 " 12 " "	64
" 1 " 2 years "	80
" 2 " 3 " "	18
Over 3 years old	8
Total	229

The positive cases occurred during the months as follows:

In	October	of 30	cases examined	10	were positive,	equal	$33\frac{1}{3}$	%
"	November	" 21	" "	2	" "	"	$9\frac{1}{2}$	%
"	December	" 19	" "	10	" "	"	52	%
"	January	" 41	" "	7	" "	"	17	%
"	February	" 36	" "	12	" "	"	$33\frac{1}{3}$	%
"	March	" 32	" "	11	" "	"	34	%
"	April	" 48	" "	9	" "	"	19	%
"	May	" 14	" "	1	was "	"	7	%
"	June			1	" "			

The table gives a list of the cases studied, with the findings. Under "positive" are recorded the cases yielding influenza-like bacilli; under "negative" are recorded the cases from which the bacilli were not obtained.

Several varieties of organisms grew on the plates, although in many cases (pneumonia, empyæma, tuberculosis) the colonies of influenza bacilli outnumbered the others. The bacteria which were present were: pneumococcus, streptococcus, Staphylococcus albus and aureus, Micrococcus catarrhalis, Diplococcus intracellularis (in a case of cerebro-spinal meningitis on the third day), diphtheria bacillus, pseudo-diphtheria bacillus, an un-

identified bacillus which was Gram negative and grew well on all culture media, and finally hemophilous bacilli resembling Bacillus influenzæ. The morphology of the last-named organisms was invariably in agreement with the descriptions of Bacillus influenzæ as given by Pfeiffer. The bacilli were sometimes polar-staining, occasionally forming threads, Gram negative, and growing only upon culture media containing hæmoglobin. When inoculated upon serum-agar, with or without glucose, no surface growth took place, but bacilli could be found in the condensation water, in which they remained alive and transplantable to blood-agar for three days. Sub-cultures from the serum-agar tubes upon fresh tubes of the same medium were unsuccessful.

It would seem from the work of Ghon and v. Preyss [2] that the influenza bacillus grows only in the presence of hæmoglobin. The amount of hæmoglobin required is, however, very small, as was shown in the course of Pfeiffer's [3] work, in which the first generation was obtained on serum-agar. Pfeiffer explained the apparent discrepancy by assuming that the sputum used for inoculation contained mere traces of hæmoglobin. Ghon and v. Preyss [2] pointed out that an amount of hæmoglobin too small to be demonstrated with the spectroscope, without the addition of hydrazin, is sufficient to insure the growth of influenza bacilli. Fichtner's [4] cultivations on sputum-agar have not been repeated by others. A small bacillus resembling the influenza bacillus but differing from it by reason of its ability to grow on serum-agar has been obtained by Jochmann and Krause [5] from cases of pertussis. The Koch-Weeks bacillus, which in some respects resembles influenza bacillus morphologically, is easily differentiated in cultures from the latter micro-organism.

In the course of this study several strains of the hemophilous bacillus obtained in cultures were compared. They came from the throat of a nurse (adult) who presented clinical symptoms of uncomplicated influenza; from the throat of an influenzal-pneumonia patient during life; from empyæmic pus; from the lung at autopsy of a case of tuberculosis with pneumonia; from the throat of a patient with measles; from the pus in a case of purulent conjunctivitis; from pertussis sputum.

In smears from the pus or sputum it was impossible to distinguish the several strains by differences in shape and size; but after several generations of growth they varied greatly in size of individual organisms and in the degree to which they formed short threads. Colonies as large as a pinhead, as described by Pfeiffer, were seen in all the strains; inoculations in

large amounts on perfectly fresh, moist blood-agar always brought about a return to the small, characteristic colonies. The very large, opaque colonies which Klieneberger[6] described for the hemophilous bacillus isolated by him from the gall bladder in a case of empyæma were not present among our strains, nor did any of our bacilli resemble in size Proteus vulgaris, as did the one noted by Klieneberger, who placed the bacillus isolated by him in the group of pseudo-influenza bacilli.

In a study of the agglutination reactions with the blood of all but two patients negative results were obtained in dilutions higher than 1 to 10. Cases of pertussis are not included in this series. The blood of the adult case of influenza reacted with the homologous bacillus in a dilution of 1 to 20 on the eighth day, and the blood from a case of intermittent influenza (see below) gave a reaction of 1 to 40 during the second and third attacks.

Rabbits were immunized with the strains indicated as "pneumonia," "tuberculosis," "adult influenza," and "eye," until their serum agglutinated the homologous bacillus in dilutions of 1 to 400. The other strains were then agglutinated by such sera in dilutions of 1 to 300 or 1 to 350, showing that the agglutinations were practically common to the group. Absorption experiments confirmed this conclusion. The following table gives the results of some agglutination tests.

Sera of rabbits immunized to influenzal-pneumonia culture:

	Dilution	1:10	1:20	1:100	1:200	1:400
"Pneumonia" culture	"	++	++	+	+	+
"Tuberculosis"	"	++	++	+	+	=
"Adult influenza"	"	++	++	+	+	+
"Autopsy pneumonia"	"	++	++	+	+	+
"Eye"	"	++	++	+	+	+
"Pertussis"	"	++	++	+	+	−

In repeating my experiments of last year[7] I found that these animals developed agglutinins much more slowly than the old ones had done, and the differentiation of the "pertussis" bacillus from the bacillus of "adult" influenza and "infantile pneumonia" was not so sharply marked as in the previous series. The agglutinins were common to the entire group, the "pertussis" strain, however, showing more frequent and greater variation in

agglutination than any of the other strains. The human blood reactions (excluding pertussis) were inactive to all strains, with the exception of the two cases mentioned above, in which the agglutination was too low for purposes of differentiation.

One bacillus which was isolated from a case of pertussis and measles-pneumonia gave reactions as high as those obtained with the "adult influenza," "eye," and "tuberculosis" strains. Whether this difference is to be explained on the ground that there was in this case a possible double infection with "pneumonia" and "pertussis" strains I am unable to say.

The absence of agglutination reactions with the serum from cases of acute pneumonia and bronchitis is to be explained probably by the fact that the agglutinins develop slowly. Rabbits require many inoculations before the agglutinins begin to appear. The blood of all the acute cases of disease was negative, the one positive reaction appearing in the case of intermittent influenza of longer standing. The positive agglutination reaction in pertussis is to be explained by the duration of the infection; and in these cases the reaction does not appear until the end of the second week of the cough.

My study on two occasions of the throats of five normal infants gave me no influenza-like bacilli whatever. Kretz found influenza bacilli in the throats of patients several months after an attack of influenza. He denominates these persons "influenza-carriers." [8] Rosenthal [9] found Pfeiffer's bacillus in the larynx and trachea in about one of six healthy persons, from which he deduced the saprophytic nature of Bacillus influenzæ. No adequate histories are given of the persons studied by him. Wassermann [10] believed that whenever influenza bacilli are found influenza exists, a view which cannot be upheld, unless perhaps in the form given by Ortner,[11] who holds that there cannot be influenza without the presence of influenza bacilli; although the bacilli may occur in the absence of the disease. Davis [12] studied the throats of twenty normal persons and found small numbers of the bacilli in two of them. They had been free from any throat disease for three months, at least, before examination, and remained well for several weeks afterwards. These cases also suffer, in interpretation, from the absence of history of possible contact with persons suffering from influenza.

The cases of broncho-pneumonia and bronchitis had been admitted for these diseases; the stage of onset, therefore, had been passed outside the hospital. The children in whom the influenza bacillus was found were distinguished by showing higher

temperature, greater prostration, more illusive pulmonary signs, and longer duration of illness than the children suffering from broncho-pneumonia uncomplicated by influenza. In the cases of pneumonia and bronchitis which developed in the hospital the bacilli were found on the second day of the disease, before marked pulmonary signs had appeared.

In the three cases of empyæma in which the pus contained the influenza bacillus, this organism had been obtained from the bronchial mucus before the operation.

Of the cases of tuberculosis twenty-four were studied, with eight positive results. Six of these gave physical signs of pneumonia. But among the sixteen negative cases, twelve gave similar signs. Eight of these cases came to autopsy: six with and two without positive findings. The question arose as to whether the presence of the bacillus influenced the clinical course of the disease. Of the positive cases five showed pulmonary consolidation, and one had acute miliary tubercles only. The last child showed as the oldest tubercular lesion a small cheesy nodule with a calcareous center in the left pulmonary apex. It seems not improbable that the influenzal infection lighted up this comparatively obsolete lesion.

Ortner [11] observed twelve persons whose sputum contained both tubercle and influenza bacilli, and he remarks that the tuberculosis alone was sufficiently extensive to have caused death. In addition he reports a case of latent tuberculosis which became active and proved rapidly fatal after infection with influenza bacilli.

Ortner [11] and Finkler [13] describe "clinically intermittent influenza." The former means an acute, the latter a chronic influenzal infection of the bronchi. I encountered one such case: B. A., 15 months old, was a rachitic child with a severe anæmia (hæmoglobin 20%); the throat was negative for influenza bacilli. Having been discharged improved, she returned in about four weeks with cough, temperature 103° F., and fine rales over both bases. On the day of admission (Dec. 28) the sputum showed influenza bacilli in almost pure culture. On January 6, the lungs were clear. On January 27, the temperature rose to 103° F., an area of tubular breathing was found, and influenza bacilli were found in large numbers in the throat. On February 3, she was improved. On February 10, a throat culture was negative for influenza. On February 14, the fever and pulmonary signs returned, and cultures were again positive. For the first time the blood gave a positive reaction (1:30) with influenza bacilli. The child had three distinct attacks of acute influenza within three months, two with physical signs of consolidation of the lungs.

Pfeiffer [3] called attention to the persistence of influenza bacilli in the lungs

of patients, but thought this especially true when the lungs are the place of diminished resistance before an attack of influenza, as in tuberculosis with cavity formation. Bronchiectasis is in this category, as is chronic bronchitis. Ortner [11] states that influenzal bronchitis may exist for months and may run an afebrile course. Lord [14] has also reported cases of long standing, and Boggs [15] observed the bronchiectatic cases. Davis [12] found influenza-like bacilli in the sputum of pertussis patients six months after the attack, and I have been able to cultivate them four months after the onset of the disease.

The influenza bacillus has been found in the nasal secretion in cases of measles by Liebscher [16] in eleven of fifty-seven persons examined during life; and in the lungs at autopsy three times. While 36 per cent. of these influenzal infected cases were fatal, the death rate among non-complicated cases was only 22 per cent. Süsswein [17] found the influenza bacillus in the nose in ten of twenty-one cases of measles and three times in the lungs at autopsy. Jehle [18] found the bacillus in the lungs in eighteen of twenty-three autopsies, and in the tonsils only once. The three writers just quoted consider that this double infection in measles influences unfavorably the clinical course. Jochmann,[19] on the other hand, finding the influenza bacillus in five out of ten autopsies in cases of measles complicated with broncho-pneumonia, believed that its presence has no such effect. Neither Davis [12] nor Klieneberger [6] expressed an opinion on this point. Albrecht and v. Preyss [20] cultivated the organism from the lungs of an infant who died of penumonia three weeks after an attack of measles.

Of the twenty-seven cases of measles which I studied nine gave cultures of influenza bacilli. The number of cases is too small to base any conclusions upon, but it may be remarked that of these nine cases, four terminated fatally, while of the eighteen negative cases seven died.

The presence of the influenza bacillus in the respiratory tract of patients suffering from measles in Europe and the United States at different seasons can hardly be considered as accidental or coincidental. As Ortner [11] has shown, influenza has never entirely disappeared from the continent of Europe since the great pandemic of 1889–90, but has become endemic (at least in the neighborhood of Vienna, from which city he writes), cases occurring every winter and spring. The same reasoning applies to New York City. The catarrhal conditions of the mucous membranes of the eyes and respiratory tract in the early stage of measles must make them favorable situations for the development of the influenza bacillus, which has, at all times, a predilection for these mucous membranes. And since the bacillus is probably always present in cities, in virtue of persistence of cases of chronic influenzal bronchitis and bronchiectasis, and

infection of the throats of persons recovered from influenza, the occurrence of influenza baccilli in a large percentage of cases of measles does not seem extraordinary. The frequent broncho-pneumonic complication of these cases due often to the influenza bacillus speaks against the view of its saprophytic nature.

In twelve cases of purulent conjunctivitis which I studied I found bacilli belonging to the influenza group. Of these, three were children dying of cerebro-spinal meningitis in which throat cultures proved negative. From these cases a terminal ward infection of the eyes cannot be excluded. Four of the cases yielded the bacilli from the throat, one from the nose and throat, and one (throat culture negative) from the lungs at autopsy. The remaining three cases recovered and yielded negative throat cultures.

That the conjunctiva may possibly be the primary seat of infection with the influenza bacillus was stated by Zur Nedden [21], who found the eye alone involved in five of thirteen persons suffering with conjunctivitis due apparently to the presence of influenza bacilli. A. Knapp [22] found this bacillus in the pus of a severe case of pseudo-membranous conjunctivitis in an infant six weeks old. No other symptoms of influenza existed, but the disease prevailed in New York at the time. Knapp [23] states that there is an influenzal conjunctivitis without other manifestations of influenza. Wynekoop [24] reports a case of very severe pseudo-membranous conjunctivitis from which the influenza bacillus was isolated in almost pure culture, there being mild general symptoms present.

One of the cases in my series is similar to the case reported by Wynekoop. It occurred in a fifteen-months-old child suffering from a pseudo-membranous inflammation of both eyes with much purulent discharge, from which the influenza bacillus was grown in pure culture. It was present also, with other bacteria in the nose. After several days a cough developed; the temperature ranged between 99.6° and 101.4° F., and influenza bacilli were found in the throat. Recovery took place in one week.

In my experience the influenza bacillus was not found in the throats of healthy children nor in the throats of children in whom lesions of the respiratory tract did not exist. When the bacilli occurred in the throat the clinical course of the disease which they complicated was, apparently, influenced by them. This fact seems to be especially true of pulmonary tuberculosis which runs a severer course when complicated by influenzal bacillus infection.

In interpreting the occurrence of influenzal bacilli in the throats of healthy persons, full histories of their previous

health and association are necessary. The influenza bacillus persists for long periods after recovery from influenza, and the relapses of the disease may well be ascribed to this condition. Besides which, healthy individuals, themselves insusceptible, may well be the carriers of the bacilli to other and susceptible persons. Hence my experience is opposed, rather, to the view that the influenza bacillus is a harmless saprophyte.

I should also like to point out that the term "pseudo-influenza" bacillus should probably be discarded. The close similarity of cultural characteristics of all the influenza bacilli has been emphasized by Neisser,[25] and my experience with the agglutination reactions leads me to regard all the strains as belonging to one family. The greatest diversity in regard to cultures and agglutination is shown by the influenza-like bacillus obtained from cases of pertussis; but even here I am not convinced that a separate and distinct species can be maintained.

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THE EFFECTS OF INTRASPINAL INJECTION OF MAGNESIUM SALTS UPON TETANUS.

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In several investigations, some [1] of which have already been published, the writers studied the effects of magnesium salts upon the animal body. The starting point for the investigations was the idea that magnesium salts may have a specific relation to the inhibitory processes of the vital functions. Among the results which have been obtained thus far may be mentioned: (1) deep anæsthesia produced in several species of animals by subcutaneous injections of magnesium salts; (2) the same effect produced with smaller doses of the salts given intravenously; (3) interruption of conductivity by local applications of magnesium salts to nerve trunks; (4) production of anæsthesia by intraspinal injection of magnesium salts. The last effect has a special bearing upon the subject of this paper and hence it will be dwelt upon at greater length.

The first experiments were made upon dogs, cats, and rabbits in which it was found that an injection of a solution of magnesium salts into the lumbar region of the spinal canal caused anæsthesia and paralysis from the pelvis down. The effects were, however, very variable. In a certain number of experiments there was no effect or the animals failed to recover from the paralysis, or succumbed to the injection. The effects became more uniform as soon as monkeys were used for the experiments. On the basis of our experiments on these animals we can state in a general way that a dose of 0.06 to 0.07 gram of magnesium sulphate to one kilo body weight injected through the fourth

[1] S. J. Meltzer and John Auer, *Am. Jour. of Phys.*, 1905, xiv, 366.
" " *Am. Jour. of Phys.*, 1906, xv, 387.
" " *Am. Jour. of Phys.*, 1906, xvi, 233.
" " *Medical Record*, 1905, lxviii, 965.
S. J. Meltzer and Haubold, *Jour. of Am. Med. Assoc.*, 1906, xlvi, 647.

lumbar intervertebral space definitely produces, sooner or later, anæsthesia and paralysis more or less pronounced, from which the animal recovers in eight to twenty hours. Repeated at proper intervals this dose caused in many of the monkeys the same general effects without recognizable harmful after-results. This dose or one a little larger caused in some instances more pronounced effects; the paralysis spread upward and the animal became, in time, generally anæsthetic. Even from these profound effects the animals recovered completely in the above-mentioned time. A still larger dose produced, in a comparatively short time, a respiratory paralysis from which the animal died without convulsions. But even in these cases artificial respiration through a tracheal tube, begun while the heart-beat was still present, would soon restore life, and if continued long enough the animal would begin again to breathe spontaneously. A dose of the salts which is just sufficient to paralyze the respiration is practically without effect upon the heart-beat and blood pressure, that is, without direct effect. To affect the heart and blood pressure much larger doses are required. This statement may be taken as the rule, from which there are a number of exceptions.

Using our experience with monkeys as the basis, spinal anæsthesia has already been employed upon human beings. It may be stated here that in human beings a dose of 0.03 gram of magnesium sulphate per kilo produces, after some time, deep anæsthesia accompanied by considerable relaxation of all the muscles, lasting from five to eight hours. The respiration is sometimes reduced, but the pulse and blood pressure remain nearly normal.

In view of the fact that the intraspinal injection of magnesium salts affected profoundly the central nervous system and especially the functions of the spinal cord, it occurred to us that certain pathological states depending upon an abnormal activity of the cord might be influenced by these injections. The first conditions to claim our attention were strychnine poisoning and tetanus. We intend in this communication to report the results obtained in our studies upon experimental tetanus.

To produce tetanus we used tetanus toxin. We had again to

contend with the difficulty of selecting proper animals. The animals which have hitherto been employed in tetanus experiments, namely mice or guinea-pigs, were useless for our study, since they could not be relied upon to bring out uniform results. Hence we resorted to monkeys. The cost of monkeys precludes their free use, and furthermore, we did not find in the literature any definite statement of the degree of susceptibility of monkeys to tetanus toxin.

Our studies are, therefore, limited in extent, and we cannot claim to have solved all the problems which the subject suggests. Nevertheless our experiments throw some light upon what we may expect from intraspinal injections of magnesium salts in the treatment of tetanus. One of the questions which seemed fundamental was: Could the intraspinal injections of magnesium salts bring about complete relaxation of the tonically contracted muscles of the tetanic state? Previous experiments had shown us that spinal injections in normal animals produced complete paralysis. But in this case we had only to deal with suppression or interruption of normal impulses, not especially of an inordinate strength. In tetanus, on the other hand, one has not only to deal with a greatly hyperæsthetic cord which, as in strychnine poisoning, causes on slight peripheral stimulation maximal clonic contractions of many muscles, but, unlike any other state, the condition is one of continued contraction of many groups of muscles which are not infrequently in a state of maximum tonicity. Hence we asked ourselves: Will the intraspinal injection be capable of conquering the extraordinary hyperæsthesia of the cord and the exaggerated tonic impulses?

Another question which we asked ourselves was whether the injections would be able to paralyze the muscles implicated in the production of opisthotonus and trismus, in other words, muscles which are innervated by cerebral and cervical nerves, without at the same time reaching the respiratory centre.

To these questions we can now give positive answers. We have seen, more than once, an animal in an exquisite state of tetanus with trismus, opisthotonus, etc., with strong hyperæsthesia and frequent clonic convulsions, lie absolutely relaxed and free from

all tetanic symptoms after receiving a single dose of magnesium sulphate (0.08 gram per kilo), while at the same time respiration, heart-beats, etc., were normal; and later the animal recovered completely from the effects of the magnesium salt. Intraspinal injections of magnesium salts are capable, therefore, of abolishing completely, at least temporarily, both tonic and clonic tetanic contractions; and certain doses also affect the muscles of the neck and face without implicating the respiratory functions.

Acting upon an hypothesis which we do not intend to discuss in this paper, we endeavored to ascertain whether these intraspinal injections are capable of retarding the development of the tetanic symptoms. To obtain an answer to this question we had to begin the magnesium injections soon after introducing the tetanus toxin. The discussion of the outcome of these experiments will be deferred until the actual protocols of some of the experiments have been given.

Protocols of Experiments.[2]

Series I. Tetanus toxin 4/100 of a mgr. fatal on the third day for 100 grams of guinea-pig. Hence it equals 1 m.l.d. for guinea-pigs. April 30, 1906, 4 P.M., injected into the right gluteal region of 4 monkeys 2 m.l.d. (8/100 of a mgr.) tetanus toxin for each 100 grams of body weight.

Monkey A, 2828 grams, control. May 2, 12 M. (44 hours after injection), animal very active, possibly slight stiffness of right leg. May 3, 2 P.M. (70 hours after injection), right hind leg definitely stiff, tail deviates to right side, seems weak, not as belligerent as formerly. May 4, 11 A.M. (91 hours after injection), found dead; arms, legs, fingers, etc., flexed and rigid. Was seen alive by the attendant one hour before.

Monkey B, female, 1698 grams. April 30. Soon after the injection of the tetanus toxin 0.4 c.c. of a 25 per cent. magnesium sulphate, = 0.06 per kilo, was injected through the fourth (?) lumbar space. Spinal fluid obtained. No recognizable effect. May 1, 11A.M. Injected into fourth space 0.45 c.c 25 per cent. magnesium sulphate. Spinal fluid obtained(?). Shortly after hind legs completely paralyzed; 9.55, P.M., completely recovered from paralysis. May 2, animal normal; no injection given. May 3, 2 P. M. (70 hours after toxin injection), right hind leg used less than other, seems somewhat rigid, tail deviates to right. Injected into fourth lumbar space 0.2 c.c. 25 per cent. magnesium sulphate. No marked effect. May 4, 11 A.M. (91 hours after injection of toxin), animal lying on side, right hind leg rigid, left leg less so, jaws closed, cannot be opened passively; arms moderately stiff, but can grasp things. 11.10 A.M., injected 0.25 c.c. magnesium sulphate into fourth (?) lumbar space.

[2] For the tetanus toxin used in these experiments we are indebted chiefly to Dr. J. J. Kinyoun who supplied us with a dry toxin.

no fluid obtained. 12 M., no definite change, perhaps jaw less locked. 2.10 P.M., injected 0.25 c.c., spinal fluid obtained. 2.15, considerable relaxation of hind legs, almost perfectly flaccid. 4.30 P.M., lies on side, moves arms occasionally, neck relaxed but unable to open jaws. May 5, 10 A.M. (114 hours after toxin injection), very rigid, arms and legs contracted, head retracted. 12.15 P.M., injected 0.25 c.c 25 per cent. magnesium sulphate into fourth space; spinal fluid obtained. 1.05 P.M., hind legs completely relaxed, head only slightly retracted, respiration slow. Jaws still locked. May 6, 1 A.M., found dead by watchman. Animal lived at least 126 hours after injection of tetanus toxin.

Monkey C, 2178 grams. April 30, soon after the injection of the tetanus toxin 0.45 c.c. 25 per cent. magnesium sulphate injected through fourth space; no spinal fluid obtained, no recognizable effect. May 1, 11 A.M., animal normal. Injected through fourth space 0.55 c. c. 25 per cent. magnesium sulphate (0.063 per kilo); spinal fluid obtained. Hind legs paralyzed within a few minutes. 11.20, unable to move, respiration slow, lid reflex active, pupils small. 11.30, respiration getting slower. 11.40, no lid reflex, no respiration, heart-beat cannot be felt. Tracheotomy made and artificial respiration started. 11.50, corneal reflex present again; heart-beat strong again. Artificial respiration continued. 3 P.M., able to breathe spontaneously when artificial respiration is stopped. Artificial respiration continued till 6 P.M., then wound sutured but tracheal canula allowed to remain in place. Animal breathes well. Left without attendance. 9.55 P.M., found dead but still warm.

Monkey D, 1708 grams, female. April 30. Soon after the injection of the toxin 0.4 c.c. of 25 per cent. magnesium sulphate injected through fourth lumbar space; spinal fluid obtained. 20 minutes later animal lying on floor of cage, moves head occasionally; respiration good. May 1, animal normal; no magnesium sulphate given. May 2, animal lively, normal. 12 M., injected 0.3 c.c of 25 per cent. magnesium sulphate into fourth space; spinal fluid obtained. Almost immediately hind legs relaxed. 1.50 P.M., animal lying flat on belly, head resting on floor, breathing very slowly but deeply, moves head slightly but apparently unable to move arms or legs. May 3, normal and fairly active. 2 P.M. (70 hours after injection of toxin), right hind leg moderately stiff, tail deviates to right side. May 4, 11 A.M. (91 hours after injection of toxin), hind legs stiff but not as marked as in "B"; able to open jaws moderately (still able to eat this A.M.). 11.15, injected 0.2 c.c. magnesium sulphate through fourth space; spinal fluid obtained. 11.30, considerable relaxation of hind legs, able to open mouth wide. 3 P.M., hind legs entirely relaxed, grasps with arms. 4.30, hind legs more rigid again, however able to open mouth (eating again) and neck fairly relaxed. May 5, 10 A.M. (114 hours after injection of toxin), lying on side, legs and arms rigid, head retracted. 12.30 P.M., 0.25 c.c. of 25 per cent. magnesium sulphate injected through fourth space; spinal fluid obtained. 1.05, some relaxation of hind legs; arms and head rigid. Injected again 0.15 c.c.; no further change. May 6, 5 A.M., found dead by watchman. Animal "D" lived about 130 hours after the injection of the toxin.

In this series of experiments in which doses of toxin used were double the minimal lethal dose for guinea-pigs, the control animal died in 90 hours and the animals B and D, which

received the magnesium, lived at least 125 hours. Furthermore, 44 hours after the injection the control monkey already showed slight stiffness of the right leg, while the treated animals showed nothing; 70 hours after injection the right leg was definitely stiff in the control while in B the right leg "seemed somewhat rigid" and in D the right hind leg was "moderately stiff."

There were also some noteworthy differences between the monkeys B and D. The first injection of magnesium had no affect at all in B, while it nearly completely paralyzed all the extremities in D. The second injection in B, which was larger and given on the following day, only paralyzed the hind legs, while in D the second dose, which was smaller than the first and repeated after forty-four hours, again paralyzed the entire animal. D was apparently more profoundly affected by magnesium than B. Corresponding with this we find that ninety-one hours after the toxin, when the control animal was already dead, the hind legs of D were stiff "but not as marked as in B" and D was able to open the jaws "moderately" while in B "the jaws were closed and could not be opened passively."

We may therefore say that in this series the intraspinal injection of magnesium salts had, apparently, a retarding effect upon the development of the symptoms as well as upon the fatal action of the tetanus toxin, and the retarding action seemed to be the more pronounced the more the magnesium salts had exercised their paralytic influence.

Series II. The same dried toxin used.

A test on guinea-pigs showed the toxin to be unaltered. July 5, injected into three monkeys 6/100 mgr. tetanus toxin for each 100 grams body weight.

Monkey A, 1935 grams; control. July 5, 4.15 P.M., injected the toxin into the right gluteal region intramuscularly. July 6 and 7 no sign of general or local effect. July 8, 11 A.M. (67 hours after injection), right hind leg stiff, "definitely stiffer than in the other monkeys"; right arm seems stiff; almost unable to open jaws. July 9, 10.30 A.M. (about 90 hours after injection), found dead; "still warm." Head retracted, jaws locked, arms flexed at elbows and wrists, pressed on body.

Monkey B, male, 2000 grams. July 5, 3.15, received toxin into right glutei. 3.25, injected through third space (?) 0.6 c.c. magnesium sulphate; no spinal fluid obtained; no effect. July 6, 2.25 P.M., injected 0.6 c.c. magnesium sulphate through third space; spinal fluid obtained. Within two minutes hind legs completely paralyzed, flaccid, no sensation; within ten minutes front legs weak, falls on side. 3.20 P.M., lid reflex active, pupils small, entire body completely relaxed.

July 7, active, no magnesium given. July 8, 11 A.M. (68 hours after the toxin), right hind leg stiff, "less than control," opens jaws wide, eats, tail slightly deviated to right. 11.20, injected through third space 0.4 c.c. of 25 per cent. magnesium sulphate. 12 M., no distinct paralysis, but lies quietly in cage. Injected again 0.2 c.c. magnesium; spinal fluid obtained. 1 P.M., very quiet, feels no pinch in hind legs but uses them to grasp. July 9, 10.30 A.M., (92 hours after toxin), head retracted, jaws moderately locked, able to open about 0.5 cm.; legs and arms stiff, unable to sit or stand. 10.45, injected 0.5 c.c. 25 per cent. magnesium sulphate between fourth and fifth lumbar vertebræ; no spinal fluid obtained. 11.40, no definite effect. Injected 0.3 c.c. magnesium sulphate between third and fourth lumbar vertebræ; spinal fluid obtained. 12.45, no marked relaxation. Injected 0.3 c.c. magnesium sulphate between third and fourth lumbar vertebræ. 1.10, legs and abdomen much relaxed, respiration slow. 2 P.M. hind legs completely relaxed, arms not so stiff, head moderately retracted. 5 P.M., hind legs flaccid, front legs a bit stiffer now, head more retracted, jaws cannot be opened passively. July 10, animal died at 10.40, A.M., 114 hours after injection of toxin.

Monkey C, female, 2540 grams. July 5, 3.45 P.M., received toxin into right glutei. Injected 0.7 c.c. 25 per cent. magnesium sulphate between third and fourth lumbar vertebræ; spinal fluid obtained. No noticeable effect, later shows marked weakness in hind legs. July 6, 2.30 P.M., monkey normal. Injected between third and fourth lumbar vertebræ 0.6 c.c. 25 per cent. magnesium sulphate; spinal fluid obtained. Hind legs paralyzed almost at once, front legs show weakness, head sinks to floor. Placed inclined, head higher in cage. 4 P.M., upper part of body more relaxed than before; lid reflex active. July 7, very quiet, moderate weakness, eats. No magnesium given. July 8, 11 A.M. (68 hours after toxin), right leg moderately stiff, less than control, eats, opens jaws wide, yawns. 12 M., injected 0.4 c.c. 25 per cent. magnesium sulphate between third and fourth lumbar vertebræ; spinal fluid obtained; shortly after hind legs paralyzed, right hind leg apparently less stiff than before; lies in cage. July 9, 11 A.M. (92 hours after toxin), able to support herself; ate this afternoon; no retraction of head; able to open jaws moderately; front legs held flexed, close to body, but are moved freely; grasps objects. Hind legs held flexed at hip and knees, moderate resistance to extension. Tail practically straight. Animal shaken by spasmodic contractions. 11.20 A.M., injected 0.7 c.c. 25 per cent. magnesium sulphate between third and fourth lumbar vertebræ; spinal fluid obtained. 11.25, no spasmodic movements so far. Suddenly respiration stopped, time lost with compression of chest, traction of tongue, etc. Later tracheotomy made and regular artificial respiration begun. No effect, animal dead.

July 9, a fourth monkey was injected with 1½ m.l.d. of the toxin used on July 5. Toxin tested on guinea-pigs and proved to have the same strength as on July 5.

Monkey D, female, 2362 grams. July 9, 12.20, P.M. received in the right glutei 1.5 m.l.d. of toxin per 100 grams. 12.26, injected 0.5 c.c. magnesium sulphate between third and fourth lumbar vertebræ; spinal fluid obtained. Some magnesium lost. 1.05, no visible effect. Injected again 0.3 c.c. magnesium sulphate; spinal fluid obtained. 2 P.M., hardly able to use legs, scratches buttocks frequently. 5 P.M., same, except that she does not scratch; does not feel

pressure of foot on tail. July 10, uses legs well. 11.30, injected 0.4 c.c. 25 per cent. magnesium sulphate between fourth and fifth lumbar vertebræ; spinal fluid obtained. In a few minutes unable to use hind legs, scratches buttocks again. 5 P.M., able to sit up but cannot use hind legs. July 11, 2.30 P.M., no sign of stiffness or paralysis. Injected 0.3 c.c. 25 per cent. magnesium sulphate between fifth lumbar and sacrum; no fluid obtained. 3 P.M., unable to use hind legs, otherwise active. 5 P.M., hind legs completely paralyzed, scratches lower parts. July 12, still scratches herself; uses hind legs; right hind leg a little stiff (11 A.M., 71 hours after toxin). 12 M., injected 0.4 c.c. 25 per cent. magnesium sulphate between fourth and fifth lumbar; spinal fluid obtained. 1.30 P.M., hind legs paralyzed and relaxed; scratches herself continually. July 13, 11 A.M. (95 hours after toxin). Right hind leg definitely stiff but can grasp with toes. Able to open jaws but not as widely as yesterday. No scratching. Injected 0.4 c.c. 25 per cent. magnesium sulphate between fourth and fifth lumbar vertebræ; spinal fluid obtained. 11.20, rests head on table, no scratching. 4.15, hind legs flaccid, uses arms freely; scratches lower parts, occasionally even bites skin. 4.20, injected 0.25 c.c. between fourth and fifth lumbar vertebræ; spinal fluid obtained. No notes on effect. July 14, 11 A.M. (119 hours after toxin), lies on left side curled up, legs flexed on hips and knees, body curved, opisthotonus, head moderately retracted, can be moved, able to protrude tongue through jaws; unable to eat. 10.40, injected 0.5 c.c. magnesium sulphate between fourth and fifth lumbar vertebræ; spinal fluid obtained. 11.45, hind legs fully relaxed, flaccid. 12 M., no pain felt in lower two-thirds of body. 1.30, lies on inclined board, head down; looks greatly relieved and improved. July 15, animal died about 6 A.M.—about 138 hours after injection of toxin.

In Series II we again see the control animal dying 90 hours after the injection of toxin, while the animals which received magnesium lived, in case of B 114 hours, and D 138 hours, while C lived only 92 hours. The last animal, however, died not of tetanus but of an overdose of magnesium while she was still in a good condition.

There was, therefore, marked difference in the development of the symptoms between the control and the treated animals. Sixty-seven hours after the injection of the toxin the control was unable to open the jaws, the forearms seemed stiff, and "the right hind leg was definitely stiffer than in the other monkeys." The other three monkeys at this time opened their jaws wide, were able to eat without difficulty, and were using their legs with only little impediment.

To recapitulate briefly, the series of eight monkeys received tetanus toxin in quantities apparently above the minimum lethal dose. Two served as controls and six were treated with magnesium sulphate intraspinal injection in varying doses and at

varying intervals. Two of the latter animals died from overdoses of the magnesium. The two controls died about ninety hours after the injection of the toxin, while four animals which received smaller doses of the magnesium survived the controls, one by twenty-four hours, one by thirty-six hours, one by forty hours, and one by forty-eight hours. In five of the magnesium treated animals the onset and the development of the tetanic symptoms were distinctly retarded in comparison with the controls. Furthermore, the retardation appeared to be more pronounced in those animals in which the injection of magnesium produced the most marked effects.

The completely relaxing effect of the injections of magnesium salts was evident in some of the injections given in these series. For reasons which will be mentioned presently we had to avoid larger doses in these series just at the advanced stage of the tetanic symptoms, and we were, therefore, frequently not in a position to demonstrate, by a single dose of the magnesium salts, the complete overpowering of all tetanic symptoms, especially at their height. But we were able to do this, as stated above, in other experiments in which magnesium was not given from the very beginning and in which the interval between injections was sufficiently long to permit the use of large doses. Here in these cases a single injection of a sufficient dose would cause in a short time absolute relaxation, and suppress completely a condition of violent tetanus.

Repetition of the injections, in the above series, brought to light an instructive fact with regard to the effects of intraspinal injections which compelled us to avoid in serial injections the use of larger doses. While a single dose of 0.06 or 0.07 gram of the salt per kilo animal would in normal monkeys produce hardly more than paralysis of the posterior extremities and pelvic region, and never proved fatal, we saw that in animal C of Series I on the second day a dose of 0.063 gram per kilo abolished the respiration within a short time after the injection; and in animal C of Series II a dose of 0.069 gram per kilo administered on the fifth day killed the animal within a few minutes, while the same dose injected on the first day had only a very

moderate effect. The fatal result was, evidently, caused by cumulative action of the magnesium salt. Some of the injected solution, apparently, remains for a time in the spinal canal or in some part of the central nervous system. At present it cannot be stated how much of the solution and how long it remains there. As the tetanus intoxication progressed we had to grope our way carefully with regard to the dose of magnesium salt to be injected as well as with regard to the intervals which had to pass between the injections. Towards the last, therefore, the doses given were often small and inefficient as we did not care to risk losing the animal through an overdose at this stage of the disease.

It is obvious that in judging the significance of our results the last mentioned fact has to be taken into account. Had we been less bound by economical considerations, and had we risked towards the end of the disease somewhat larger doses at more frequent intervals, we feel confident that we would have been successful in some cases in prolonging the life of the animal by many more days, although we might have killed some monkeys prematurely by an overdose of magnesium. With regard to Monkey D in Series II, for instance, we feel confident that had we given a slightly larger dose on the seventh day and repeated the dose of magnesium also on the evening of that day, the animal would not have died on the eighth day following the injection of toxin. Furthermore, it is possible that in some cases we might even have been successful in tiding over the animal until the newly formed antitoxin could have overtaken the balance of free toxin, and the metabolic processes of the body have mastered the toxin fixed by the nerve cells; or in other words we might have saved the animal's life.

While, therefore, as far as this short series of experiments is concerned we were unsuccessful in saving, by means of intraspinal injections of magnesium, the lives of any of the animals poisoned with tetanus toxin, the results do not justify the conclusion that such intraspinal injections are incapable of saving tetanic animals. On the contrary, the experiments brought out certain rather encouraging facts. It was established that the intraspinal

injections are capable of abolishing completely all tonic and clonic muscular contractions, thus eliminating for many hours at a time one at least of the most dangerous factors in the disease. Moreover, the experiments disclosed the important fact that intraspinal injections of magnesium salts are capable of retarding perceptibly the progress of the disease.

The last fact is a new and, in a certain sense, an unexpected point, inasmuch as the retardation of the progress and development of the tetanic symptoms cannot be considered simply as a consequence of the inhibitory property of the salts or of their ability to relieve the symptoms. This effect was, however, not wholly unexpected by us, since, as we stated above, on the basis of a certain hypothesis which we had formed, we looked for it, and, therefore, in a part of the experiments we began to inject the salts long before there appeared tetanic symptoms to relieve. As regards the hypothesis itself, we believe it would be premature to state it here; we intend, however, to discuss it shortly in connection with another investigation.

We mean to mention in this place some distinctions with reference to our problem between human beings and monkeys which might have theoretical as well as practical significance. There is in the first place a difference in the two species of extension of the spinal cord in the lumbar canal. The autopsies on monkeys taught us that in the species which we employed the cord extends to the sacrum. Any injection made, therefore, in the lumbar region could produce injury of the cord. It is, however, noteworthy that every monkey except one (an animal which had been used previously for other experiments) showed, nevertheless, no effect due to this cause, although in some cases repeated injections were made. Yet it cannot be excluded that in some cases a part of the fluid may have entered and remained in the cord, a possibility which might perhaps account for variation of effect where an identical dose of the salt was employed under apparently the same circumstances. In human beings, on the other hand, the injections are always made below the cord.

Another point of distinction arises from the circumstance that in monkeys only very little spinal fluid can be obtained and none

of the injected fluid could be recovered, even soon, after the injection. When intraspinal injections are made in human beings, spinal fluid equal in amount to the injected quantity of the salt solution can be removed previously. This preliminary lowering of the pressure within the canal aids possibly in a more rapid distribution of the solution through the entire canal, and is perhaps partly responsible for the observed fact that in human beings the effective dose of the magnesium salt (per kilo body weight) is not even one-half that which is required for monkeys. Furthermore, in human beings "washing" of the canal proved to be a helpful procedure. When the respiration became too slow spinal fluid was withdrawn and saline was injected several times and again withdrawn. The patient soon recovered from the effects of the magnesium salts. In monkeys we were deprived of such a help in case of danger and had, therefore, to keep away from the danger line.

Intraspinal injections of magnesium sulphate have already been employed in a small number of cases of human tetanus. We shall append here brief notes, as far as they have come to our notice, concerning these cases.

Case 1, described by Dr. J. A. Blake.[3]—A boy of fifteen had trismus, opisthotonus, etc., and a temperature of 104.4 F. This was on the sixth day after the first tetanic symptoms appeared, which began seven days after injury to the fingers, and he had already received 115 c.c. of tetanus antitoxin and morphine without relief. The boy's weight was about 65 kilos and 4.5 c.c. of a 25 per cent. solution of magnesium sulphate was injected into the spinal canal, that is 0.02 gram per kilo. "Six hours after the injection the jaws could be opened wide; the stiffness of the neck and back had disappeared . . . and the temperature had fallen to 102.6 F, the pulse to 104; the respirations were 14." Thirty-three hours after the injection of magnesium sulphate its effect seemed to have entirely passed off and another injection of the same amount was made. "In two and a half hours he was relaxed and asleep." After twenty-four hours there was a steady increase in the spasmodic contractions and after thirty-seven hours the opisthotonus returned. Another injection was then given of 8 c.c. of 12½ per cent. magnesium sulphate. This injection was again followed by complete relaxation. A similar injection to the last was repeated after twenty-nine hours with a similar result. In the next four days "the symptoms were not severe and the patient was kept comfortable with morphine and chloral." On the sixth day after the last injection "the opisthotonus became more severe

[3] J. A. Blake, *Surgery, Gynecology, and Obstetrics*, 1906, v, 541.

and there was considerable rigidity of the jaw muscles and considerable pain," so that a fifth injection of magnesium was given which was followed by marked relief. After this the convulsions were much less frequent and severe, and gradually disappeared, so that after fourteen days the boy was practically well.

In this case with complete recovery five intraspinal injections were given without any injurious effect whatsoever. Each injection produced complete relaxation lasting quite a long time. There was no evidence of a cumulative effect, but, on the contrary, "a certain amount of tolerance seemed to be established for the sensory impulses and the cerebrum . . . the motor symptoms were practically the same after each injection." No rise of fever occurred after the last three injections.

Case 2, boy reported by Dr. Samuel Logan,[4] New Orleans.—Tetanus set in on the ninth day after an injury of the hand inflicted by a toy pistol. On the third day of the disease, after antitoxin and chloral and bromide had been given in full doses with no avail, 4 c.c. of a 25 per cent. solution of magnesium sulphate were injected into the spinal canal—1 c.c. for every 20 pounds of body weight—under anæsthesia. It was noticed "that under complete anæsthesia the abdominal muscles were never relaxed, the abdomen being rigid and hard as a board on palpation." Forty minutes after the injection all the muscles of the body were completely relaxed, the abdomen was soft, and the mouth could be opened readily; the patient was perfectly conscious and said that he felt well. About five hours after injection a profuse bronchorrhœa developed and the breathing grew rapid and shallow. Atropin seemed to have relieved this condition perceptibly. Pulse was 140 and thready. (On the day before the injection the pulse varied between 120 and 140 and the respiration between 24 and 36.) About twelve hours after the injection the tetanic symptoms began to come back, and eleven hours later the muscles of head, neck, and abdomen were rigidly contracted, but the general convulsions did not return. Temperature 102 F., pulse 150 and thready; respiration 30. Under anæsthesia a small dose of magnesium sulphate (50 minims of a 25 per cent. solution) was again injected. Thirty-five minutes after the injection, all the muscles of the body were again completely relaxed. Three hours after injection bronchorrhœa again developed which was again partially relieved by atropin and applications of heat. The general condition of the patient, however, gradually became worse; the pulse rose to 192, the respirations to 50, and the patient died in a state of complete relaxation about sixteen hours after the last injection. No autopsy was made.

In this case too the intraspinal injections of the magnesium abolished promptly and completely all tetanic symptoms. The

[4] Samuel Logan, *Journal Am. Med. Assoc.*, 1906, xlvi, 1502.

bronchorrhœa was probably no after effect of the magnesium injection, but possibly of some complication (pulmonary tetanus?); the high pulse and respiration before the injection of magnesium would seem to indicate this.

Case 3, observed by Dr. Charles D. Peck, at the French Hospital, New York. We are enabled, by the courtesy of Dr. Peck, to give the following notes.—A man thirty-five years old received an insignificant wound in the sole of the foot, which healed rapidly. Four days after the injury a violent tetanus broke out. He received large doses of antitoxin and sedatives but no relief. An intraspinal injection of magnesium sulphate, 1 c.c. of a 25 per cent. solution to every 25 pounds of body weight, had only a moderately relaxing effect. After twenty-four hours the injection was repeated but this time 1 c.c. of the solution was given for every 18 pounds of body weight. The anæsthetizing and relaxing effect was now complete. We had the privilege of seeing the patient about four hours after the second injection was made. He was under perfect general anæsthesia; all parts of the body were completely relaxed, the abdominal muscles being especially soft; no sign of tremor or twitch anywhere except now and then a slight retraction of the right corner of the mouth produced by tickling the nasal mucous membrane. Lid reflex was present, respirations 22 per minute, fairly deep. Pulse 120, of fair quality, and temperature 102 F. The temperature and pulse were the same as before the injection. Three hours later the patient was given an intravenous injection of 60 c.c. antitoxin. The patient died suddenly about two hours later. Dr. Peck informs us that the respiration remained good to the end.

These three cases show that in human beings the intraspinal injection of magnesium sulphate is capable of abolishing completely for quite long periods the severest symptoms of tetanus. In the first case it was apparently the means of saving the patient's life. That the other two patients died while their muscles were in a perfectly relaxed condition may be taken to indicate that tetanus toxin has other poisonous effects besides those which produce the tetanic symptoms. Since, however, other interpretations are possible, we cannot afford to enter here into a discussion of this subject.

CONCLUSIONS.

Intraspinal injections of magnesium sulphate, in doses which do not affect the respiratory centre or other vital function, are capable of abolishing completely all clonic convulsions and tonic contractions in cases of human tetanus, and experimental

tetanus in monkeys. The relaxing effects of the injections may last twenty-four hours or longer.

In experimental tetanus in monkeys early intraspinal injections of magnesium salts are capable of retarding the progress and development of the tetanic symptoms.

THE OCCURRENCE OF SPECIFIC IMMUNITY PRINCIPLES IN THE BLOOD OF VACCINATED CALVES.*

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The immunity which human beings develop towards vaccinia, after a successful vaccination, is the classical example of an artificially induced active immunity. The duration of the immunity to vaccinia is variable but extends frequently over many years. Many animals are subject to inoculation with vaccinia and they show, as in the case of man, a state of immunity following the disappearance of the successful vaccination. The calf, as is well known, is now generally employed for the production of the vaccine-virus which is used to protect human beings from smallpox. At one period in the development of specific serum therapy an attempt was made to utilize the immune principle which it was presumed existed in the blood of animals which have passed through a successful vaccination. The hope was entertained that, possibly, the serum of the vaccinated calf might exercise curative properties upon persons suffering with smallpox. This hope has not been realized. In order to prove the existence of immunity principles in the blood of vaccinated animals the attempt was made to protect, by inoculation of the blood of these calves and monkeys, fresh animals from vaccinia. In spite of some successful results the experiment may be said to have often failed.[1]

The failure of the blood of vaccinated animals regularly to confer passive immunity or to exert curative effects cannot be held to disprove the existence in the blood of immunity substances. Tests of the kind mentioned are exceedingly gross. Moreover,

* The work reported here was begun in the Pathological Laboratory of the Johns Hopkins University and completed in the Rockefeller Institute for Medical Research.

[1] The literature covering this subject is reviewed by Sternberg and Reed, *Trans. Amer. Assoc. Phys.*, 1895, p. 57 and by Copeman, Vaccination, London, 1899.

in the case of vaccinia and variola we labor, at present, under the great disadvantage of being ignorant of the nature of the specific microorganisms of the diseases. Once in our hands it is probable that these microorganisms would quickly yield evidences of antibody or immunity-principle formation. Hence the question arises whether by any other method than has thus far been employed the existence of antibodies can be shown for vaccinia and for smallpox. Should it be possible to demonstrate, by an accurately quantitative method, the existence of antibodies in one of these diseases it would not only be safe to assume the existence of antibodies in the other, but the way would be opened to ascertain whether the immunity principles of vaccinia and smallpox are qualitatively identical. I have succeeded, by the application of the Bordet-Gengou method of fixing hæmolytic complement, in proving the existence of antibodies in the blood serum of vaccinated calves; and I am engaged upon the study of smallpox with the same method. Should this second investigation yield positive results a new diagnostic measure for this disease would be available.

As long ago as 1901 Bordet and Gengou [2] showed that if fresh guinea-pig complement was added to typhoid, cholera, plague, or anthrax bacilli which had been treated previously with their specific anti-sera, the complement would be bound or absorbed by the bacilli. The test of the union of complement and bacilli was made with sensitized rabbits' erythrocytes, which remained intact under these conditions, but underwent hæmolysis in the control experiments in which the anti-sera were omitted. By means of this method the existence of antibodies was demonstrated in the serum of a horse immunized to plague bacilli, and in the blood of two persons who had recently recovered from typhoid fever. Malvoz [3] in the same year employed this method to show the presence of antibodies in the blood of animals inoculated with cultures of yeast. The later work along this line, through which antibodies for several microorganisms in animals, and in the blood of persons suffering from various diseases, have been demonstrated, dates from Moreschi's [4] experiments on the combination of complements and specific immune sera and Neisser and Sachs' [5] application of the principles of these methods to the detection of traces of blood in medico-legal practice.

The credit of using practically the method of complement absorption for the detection of antibodies in infectious diseases must be given to Wassermann and

[2] Bordet and Gengou, *Annales de l'Institut Pasteur*, 1901, xv, 289.
[3] Malvoz, *Annales de la Soc. Med.-Chir. de Liege*, 1901, xl, 275.
[4] Moreschi, *Berliner klin. Woch.*, 1905, xlii, 1181.
[5] Neisser and Sachs, *Berliner klin. Woch.*, 1905, xlii, 1388.

his pupil Bruck,[6] since in the hands of Bordet and Gengou no general application was made of their discovery. But the revival of the method followed from Moreschi's and Neisser and Sachs' experiments. Wassermann and Bruck[7] employed it to demonstrate, as they thought, the existence of tuberculin in tuberculous foci, and Wassermann, Neisser and Bruck[8] and Detre[9] believed that by its means they discovered the existence of antibodies in syphilis. Kolle and Wassermann[10] have applied the method successfully to the estimation of the quantity of immune bodies which exist in the anti-serum prepared in the horse for the diplococcus of epidemic meningitis.

On account of the similarity of methods used for the study of syphilis by Wassermann, Neisser, and Bruck, and vaccinia by myself, I will give briefly the method employed by the former, prefacing what I say by the statement that my experiments were well advanced when their paper on syphilis appeared. Although the specific organism of syphilis has now probably been discovered, yet so long as it cannot be cultivated and obtained in mass, resort must be had to pathological products which contain the microorganism in numbers large enough to yield a specific extract of them, or an extract, at least, containing the specific products of their growth and metabolic activities. They employed for extraction condylomata and the organs of a still-born syphilitic child; and for the immune bodies they used the inactivated blood serum from cases of syphilis. Wassermann, Neisser, and Bruck found that when these extracts were combined with the inactivated syphilitic serum and fresh guinea-pigs' serum, the mixture having been placed in the incubator for one hour, that the subsequent addition of sensitized corpuscles, which in the control tubes underwent hæmolysis, were in the other tubes still intact at the end of twenty-four hours.

For the purposes of the studies on vaccinia the vaccine virus and serum of vaccinated calves were obtained through the courtesy of the H. K. Mulford and Parke and Davis companies, to whom I wish to express my indebtedness. I wish to thank, especially, Dr. J. J. Kinyoun of the Mulford Company for repeated acts of kindness. The normal sera of calves and oxen were obtained

[6] Wassermann and Bruck, *Med. Klinik*, 1905, i, 1409.
[7] Wassermann and Bruck, *Deutsche med. Woch.*, 1906, xxxii, 449.
[8] Wassermann, Neisser, and Bruck, *Deutsche med. Woch.*, 1906, xxxii, 745.
[9] Detre, *Wiener klinische Woch.*, 1906, xix, 619.
[10] Kolle and Wassermann, *Deutsche med. Woch.*, 1906, xxxii, 609.

directly from slaughter-houses. The inactivation of all the sera was conducted in a water-bath, at 56° C., this temperature being maintained for one half-hour. The virus was ground finely and suspended in 0.85 per cent. salt solution. The preservation was made with 0.4 per cent. carbolic acid or with toluol. The emulsions thus obtained were shaken for three or four days in the mechanical shaker and then subjected to centrifugalization until clear, the fluid portion being decanted from the sediment. This extract was used in the experiments.

Each sample of extract was tested (1) for hæmolytic action on blood corpuscles, and (2) for its absorbing power for complement. All the extracts when used in relatively large amounts caused hæmolysis, an action which is to be attributed, probably, to the effects of tissue-products described by Metschnikoff [11] and Korschun and Morgenroth.[12] As a rule one cubic centimeter of the vaccine-extracts produced in twenty-four hours complete hæmolysis of one cubic centimeter of 5 per cent. washed hens' corpuscles, and 0.8 cubic centimeter of the extracts produced partial hæmolysis. The extracts when used in large quantities also combined or neutralized the action of complement.

An amount of the extract as large as 0.6 cubic centimeter would, if permitted to act for one hour at 37° C., annul the hæmolysis of washed corpuscles and inactivated serum added subsequently. Hence in every experiment the action of the extract, either in producing hæmolysis or in annulling it by fixation of complement, was carefully excluded through the employment of sufficient controls.

The hæmolytic serum was obtained by inoculating rabbits with washed hens' corpuscles, and the hens' corpuscles used for this purpose and for the hæmolytic tests were washed, after removal of the serum, three to four times with salt solution. As complement fresh guinea-pigs' serum was employed, the amount required being 0.05 cubic centimeter.

The quantities of each serum required to bind the complement in the presence of the vaccinial extract was determined, but in

[11] Metschnikoff, *Annales de l'Institut Pasteur*, 1899, xiii, 737.
[12] Korschun and Morgenroth, *Berliner klin. Woch.*, 1902, xxxix, 870.

the table only the smallest quantity is given. The method of conducting the experiments was to mix the extract, calf's or ox's serum, and complement and add salt solution to bring the volumes uniformly to two cubic centimeters. The tubes were then placed in the thermostat for one hour, after which the hens' corpuscles and rabbits' immune serum, the latter diluted uniformly to 0.5 cubic centimeter, were added. After shaking to secure mixture of all ingredients, the tubes were returned to the thermostat for two hours, and then placed in the refrigerator for eighteen hours. The accompanying table gives in brief the results of the experiments. The results were, however, repeated many times with different extracts and sera of vaccinated calves.

TABLE

Immune serum No. 1	Immune serum No. 2	Normal calf serum	Normal ox serum	Guinea-pig complement	Vaccine extract	Anti-serum (hen)	10% hens' corpuscles	
0.05				0.05	0.3	0.01	0.5	0
	0.033			"	"	"	"	0
		1 c.c.		"	"	"	"	+
			0.7 c.c.	"	"	"	"	+

Controls.

				0.05	0.5	0.01	0.5	+
0.5				"	0	"	"	+
	0.5			"	0	"	"	+
		1 c.c.		"	0	"	"	+
			0.7	"	0	"	"	+
0.5				0	0	0	"	0
	0.5			0	0	0	"	0
		1 c.c.		0	0	0	"	0
			0.7	0	0	0	"	0
				0.05	0	0.01	"	+
					0.5	0	"	0
						0.01	"	0

As the tabulated results show, the quantity of immune bodies present in the serum of vaccinated calves is small, but since no bodies acting in the same manner as these were ever detected in the serum of non-vaccinated calves, and no inhibition of hæmolysis was produced by ox serum when used in amounts below 0.8 cubic centimeter, the conclusion is warranted that the serum of animals which have recovered from vaccinia contains specific antibodies capable of uniting with certain specific substances contained in extracts of vaccine pustules. In so far as our present knowledge permits us to account for the peculiar complement binding described above, we are justified in regarding the antibodies of the serum of vaccinated calves as of the nature of immune bodies. It is hoped that the results of the study of vaccinia may soon be extended to the study of variola.

The work described in this paper was begun in the Pathological Laboratory of the Johns Hopkins University. I wish, therefore, to express my indebtedness, for many courtesies, to Professor Welch and Dr. Ford.

AN EXPERIMENTAL RESEARCH INTO THE RESUSCITATION OF DOGS KILLED BY ANESTHETICS AND ASPHYXIA.

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PLATES XLII–XLIX.

In a previous communication by one of us (Crile) resuscitation was attempted by means of both direct and indirect cardiac massage, with and without artificial respiration, with and without intravenous saline infusion, and with and without the addition of adrenalin. The results of these experiments may be summarized as follows: By cardiac massage alone animals were rarely resuscitated at any time after quiescence of the circulation and respiration; by combining either direct or indirect cardiac massage with artificial respiration and the head-down posture a certain percentage of the animals were recovered after the lapse of from one to three minutes. The results, however, were quite uncertain, and in the case of death from chloroform recovery was the exception. By adding to cardiac massage artificial respiration and intravenous saline infusion, resuscitations were in a slightly greater proportion successful. The same procedures, with the addition of adrenalin to the intravenous saline infusion, were markedly more successful in the deaths from asphyxia through rarely so in the cases of death from chloroform. It required in almost every instance a vigorous compression of the thorax over the heart for a considerable time, varying from five to ten minutes, before resuscitation could be accomplished. Even then there were a considerable number of failures. In the majority of these failures autopsy showed the heart to be in a state of marked distension, indicating complete cardiac paralysis; not infrequently when results were delayed, the acute cardiac dilatation was partly relieved by venesection

and by placing the dog in the inclined head-up posture, thereby partly relieving the paralyzed heart of its over-distension.

In this research the problem was approached from the arterial side, with the following physiologic factors as a basis: The physiological researches of Ringer, Kuliabio, Sollman, and others had shown that the excised heart could be made to beat again, even many hours after excision, when the coronary arteries were subjected to a considerable pressure from some circulating medium. The researches of Sollman showed that the *inauguration* of the beat was more dependent upon the physical factor of the increased pressure in the coronary arteries than upon the quality of fluid producing such pressure. He was able to inaugurate cardiac beats by perfusing the coronaries with metallic mercury. The basic problem, then, in resuscitation seemed to us to be that of securing by means of some infusion a coronary pressure approximately amounting to from thirty to forty millimeters of mercury. It was impossible to raise the coronary pressure in the intact animal to this height by means of cardiac massage alone; it was almost impossible to raise it by the mechanics of infusion alone, and generally impossible to raise it to this height by means of cardiac massage and plain infusion combined.

The value of adrenalin in raising the blood pressure, by its action upon the vascular walls in the state of suspended animation, has been heretofore thoroughly established. Introducing the adrenalin into the venous circulation, while easy and practical, had the following disadvantage: the adrenalin first came in contact with the vessels having the least power of influencing the blood pressure, and before a material rise could be effected by its action upon the arteries it was necessary that the solution should pass through the right heart, the lungs, then back to the left heart on its way to the aorta, thence affecting directly the coronary artery. In the previous research it was found that this too often caused an accumulation of solution and blood in the dilated, paralyzed chambers of the heart, defeating resuscitation.

It seemed reasonable to us to suppose that the most direct and effective way of producing a coronary pressure amounting to thirty or forty millimeters of mercury was by introducing a

solution of adrenalin into the arterial system toward the heart. In this way the moment the adrenalin was introduced it caused a contraction of the strong arterial walls, beginning to produce an arterial pressure which would communicate directly with the coronary artery without first passing through the already distended and paralyzed chambers of the heart and through the lungs. These considerations were particularly impressed upon us by a clinical case which has elsewhere been reported.

Sixty experiments were made in which the tracings were preserved and the records noted. In the recovery experiments the greatest care was taken to secure for the animals all the comfort possible, and as few of this class of experiments were made as was consistent with the collection of data sufficient for our conclusions.

Three groups of cases were investigated: (1) death from asphyxia, (2) from ether, (3) from chloroform. Inasmuch as the methods and results were quite similar, they may all be described together.

We first tested the method as to its usefulness in raising the blood pressure, and found that by tying a cannula into an artery toward the heart and introducing through it a solution of adrenalin the blood pressure was immediately raised, the latent period being distinctly less than when the solution was introduced intravenously. In fact, we may say that there was no latent period, the blood pressure rising instantly. It was found, too, that the most effective method of raising the blood pressure was by the injection of the full strength of 1–1000 solution of adrenalin by means of a hypodermic syringe into the rubber tubing near the cannula. The results in these experiments showed a distinctly more powerful action than in the experiments in which the solution of adrenalin was previously made and poured into the funnel.

The first experiment was made upon an animal killed by chloroform. A cannula was inserted in the femoral artery directed toward the heart. After two minutes artificial respirations were begun and the saline solution was allowed to flow for perhaps ten seconds; then a hypodermic injection of from one to two cubic centimeters of 1–1000 solution of adrenalin was given into

the tube near the cannula. A few seconds later the blood pressure began to rise steadily; then a few firm pressures upon the thorax over the heart caused a leaping up of high pulse waves, and at the end of three quarters of a minute the heart beat vigorously, driving the blood up into the infusion bottle, which was raised to a height of five feet. The saline injection and cardiac massage were discontinued, and in a few minutes irregular respirations slowly began, decreasing in force and increasing in frequency until the normal was established. The animal was then killed.

Following this experiment others were made, each time a longer period being allowed to elapse from the moment of death until the beginning of resuscitation. In determining the moment of death from chloroform it was found that by administering an excessive dose by inhalation the blood pressure sank with remarkable rapidity to the abscissa line, but that after this in forty per cent. of such deaths feeble pulse waves reappeared after periods varying from a few seconds to over three minutes, the blood pressure rising to from ten to thirty millimeters, then gradually declining, the pulse wave progressively diminishing in length. After a period of resumption of beat varying from a few seconds to six minutes, and averaging two minutes, the pressure usually fell to the abscissa line and no further waves were recorded upon the drum. It was only after the pressure had reached the abscissa line, after all pulse waves had ceased, and no heart sound, however feeble, could be auscultated, that we recorded the moment of death. The time was then extended to five, ten, fifteen, twenty, and thirty minutes in adult dogs, and in puppies up to an hour.

The limitations of the method soon appeared. Among them were the following: (a) ante-mortem clotting, (b) over-distension of the heart, (c) later failure of the heart, circulation, and respiration, (d) the apparently imperfect recovery of the brain.

Ante-mortem Clotting.—When a considerable time had elapsed, say ten minutes or more after death, and particularly if there had been some delay in resuscitation of the heart, thereby inducing vigorous and protracted massage, clotting occurred more frequently. Direct cardiac massage was then made, which seemed

further to facilitate clotting. In other cases indirect massage was made with extreme vigor, showing like results. Finally, an animal was etherized to the point of respiratory failure, then sustained with a very low blood pressure by means of artificial respiration combined with vigorous cardiac massage, for a period of about seven minutes, after which the heart was opened before it had ceased beating and small clots were found. No infusion in this case had been given and no other operation was performed. This seemed to confirm our previous conclusion that either vigorous or prolonged massage of the heart predisposed to ante-mortem clotting and that direct massage of the heart favored clotting more than indirect massage by rhythmic pressure upon the intact thorax over the heart.

It was found that animals could be resuscitated after several compressions of the chest by waiting a little longer for the rise in the pressure from the adrenalin infusion alone. It was obvious that in our previous experiments we gave unnecessary massage as to force, frequency, and duration. This is the more reasonable since the object of the massage is not that it will materially aid the heart in *inaugurating* its rhythmic contractions, but that it will distribute the fluid through the circulation which has been injected by gravity alone. Pressing upon the thorax itself, would effect a certain amount of circulation, if no heart existed and a system of vessels, one having valves and the other not, replaced it, and in the dead animal pressure upon the thorax alone is capable of producing an artificial circulation. This is by no means accomplished by its action upon the heart solely, but by its action upon all the large vessels—arteries, veins, and capillaries together.

We also observed what on *a priori* grounds we supposed to be true, namely, that a tendency to clotting was increased in a direct ratio with the lapse of time. When clotting occurred resuscitation was practically always impossible. The heart would beat for a short time, but the beat would soon fade away.

Over-distension of the Heart. — In the unsuccessful cases, particularly those in which a considerable amount of infusion was given, the heart at autopsy was found over-distended. This

over-distension was due to two factors; the first was the inevitable accumulation of blood in the heart during its gradual failure either in asphyxia or following chloroform and ether; second, the infused fluid passed through the coronary arteries, through the coronary vein into the right side of the heart. The left heart was not generally so distended as the right heart. It is obvious that if for any reason resuscitation is not promptly made the accumulation of fluid in the right heart must not only be considerable but must occur under pressure; since the valves prevent the escape of the fluid toward the periphery of the veins the accumulation must be largely in the heart itself, and when this condition is reached a peculiar vicious circle has been established. The greater the efforts at resuscitation by increasing the infusion the greater the distension of the paralyzed ventricle and the more certain is the failure of the attempt. In some instances after the chest was opened and the heart on inspection was found distended, rhythmic contractions of the heart began; on opening the pericardium in one instance a splendid resuscitation (Fig. 6.) was made.

Later Failure of the Heart, Circulation, and Respiration.—In a number of instances after a temporary resuscitation the circulation and the respiration failed, after which a second attempt at resuscitation was useless. In these cases the failure was generally gradual and occurred after the lapse of a varying period of time, ranging from three and a half to twenty hours. In these cases for the most part ten minutes or more had elapsed before resuscitation was attempted. A few animals in which resuscitation was attempted in less than ten minutes also showed later failure. We are not prepared to state the exact cause of this later failure.

The Apparently Imperfect Recovery of the Brain Proper.—In resuscitations after ten minutes, particularly those after from fifteen to twenty minutes, it was noted that while the circulation and the respiration were satisfactory the animal did not regain consciousness; the reason for this we are not at present prepared to state, but it will be further investigated.

We also investigated the influence of prolonged chloroform

anesthesia prior to death. Animals were kept under chloroform anesthesia for an hour and then killed by an over-dose. They were resuscitated perhaps a little less readily than the animals killed immediately by an over-dose, though their resuscitation was, up to ten minutes, satisfactory.

Hirudin.—After encountering the difficulty with clotting in the delayed cases, we tested the value of hirudin in the prevention of clotting. In the cases in which hirudin was given prior to death no clotting occurred in a single instance in the heart or blood-vessels. In these cases, which were limited to puppies not over three months old, we are, however, inclined to believe that œdema of the lungs was distinctly more marked. The blood seemed to lose its viscosity and the œdematous fluid accumulated rapidly in the bronchial tubes, and finally flowed out of the trachea, presenting a reddish tinge. In the successful cases this always occurred before the inauguration of the heart-beat. There was in addition free blood-tinged fluid in the thorax. While hirudin, in the case of puppies at least, entirely obviates the clotting factor it introduces an equally fatal obstacle, *i. e.*, waterlogging of the thorax, which we were not able to prevent by reducing the infusion pressure to a minimum consistent with its efficacy.

Puppies.—The experiments on puppies showed at once that resuscitation was more readily done, and successful after a longer period after death, than in the adult dog. In three instance thirty-five minutes after death a fairly good circulation was obtained (Fig. 8).

Solution Employed for Infusion.—The observations in this series as well as in other researches within the past year have led us to conclude that Ringer's solution yields more favorable results than saline solution. The most satisfactory results were obtained with the infusion bottle at a height of five or six feet.

Conclusions.—Based on the data supplied by these researches and those of preceding researches on similar lines we arrive at the following conclusions:

(1) Animals after death from chloroform, ether, or asphyxia up to five minutes were uniformly and readily resuscitated,

provided that the full techinque which has been described was applied; up to ten minutes there was an occasional failure; beyond ten minutes consciousness was rarely restored, and the proportion of successes in the resuscitation of the circulation and the respiration diminished with the lapse of time; after twenty-three minutes in adult dogs and thirty-five minutes in puppies complete return of the circulation was not accomplished.

(2) After death from chloroform and ether animals were more readily resuscitated than after death from asphyxia.

(3) Resuscitation, if successful, occurred within one minute after the administration of adrenalin in the majority of instances; it rarely occurred after an interval greater than three minutes.

(4) The greatest difficulty to be overcome was clotting. The probability of clotting increased in direct ratio with the lapse of time after death. Cardiac trauma predisposed to clotting.

(5) The younger the animal, up to certain limits, the more readily it was resuscitated.

(6) Any artery may be used for the infusion, though the advantage is probably slightly in favor of the carotid. The infusion should be directed toward the heart.

(7) The probable success of resuscitation is greater in inverse relation to the lapse of time after death; a rapid rise in the arterial pressure is attained by arterial infusion with a therapeutic dose of adrenalin, together with good artificial respiration and the avoidance of unnecessary cardiac trauma by massage. All artificial aids should cease as soon as the functions are competent.

ILLUSTRATIVE PROTOCOLS.

EXPERIMENT IV. *The effect of time and of massage on the formation of clots.* Mongrel mastiff, 20 K. Chloroform administered until death.

10.09—Respiration stopped.

10.14—Pulse was not palpable at carotid.

10.15—Heart sounds ceased. The opened carotid bled for some time with a non-pulsating drop flow which stopped when the head was raised. A tracheal cannula was rapidly adjusted, and with a manometer cannula in the left carotid a small cannula in the right carotid was connected with the saline infusion bottle at a height of seven feet. A pin-hole cannula in the left femoral vein was connected to an infusion bottle containing 1–100000 solution of adrenalin chloride at a height of three feet.

10.47 (32 minutes)—Resuscitation attempted. Cardiac massage was vigorous. Two cubic centimeters of the solution of adrenalin chloride were injecte

into the infusion tubing. No results came from the first dose of adrenalin within two minutes. With a second dose there was a perceptible manometer pulse and measures were temporarily stopped, the blood pressure being 40 mm., but the level quickly declined to the abscissa. Further efforts, namely massage and a mass dose of adrenalin with a continuous 1–100000 infusion into the femoral vein, failed to improve the heart-beat and to raise the blood pressure. After some time, when no pulse could be felt, the carotid infusion was again started and a second time beats were evoked, but other measures failed to keep up the heart's activity. In the latter part of the experiment the dog's feet were lowered.

At autopsy the heart was full of bloody fluid, the pericardium was tense and the heart dilated. Clots were found in the heart.

EXPERIMENT XXVIII. *Resuscitation six minutes after death.* (Fig. 1.) Ether anesthesia. This experiment was performed with moderate aseptic precautions.

10.59—Control blood pressure 156 mm.

11.00—Began to force ether.

11.04—Respiration stopped, after which cardiac failure gradually ensued.

11.06—Heart sounds ceased and pulse not palpable.

11.12—Resuscitation was begun (6 minutes). Saline infusion plus 2 c.c. of adrenalin chloride (1–1000) was administered through the axillary artery together with artificial respiration and gentle heart massage. About twenty respirations were given. The heart began to beat in about one and a half minutes. The blood pressure rose sharply to 240 mm., which level was sustained for several minutes. It gradually fell to 80 mm., at 11.24.

11.18—Spontaneous respiration began.

11.20—Ether was required and given. The respiration was at first very irregular and spasmodic, gradually becoming quiet and regular.

11.29—Blood pressure 130 mm.

11.40—Blood pressure 160 mm.

11.50—Blood pressure 160 mm. The trachea was sewed up, the wounds in the neck and axilla were closed with large gauze drainage and were bandaged. The dog was removed from the table and allowed to recover. Late in the afternoon the dog had considerable respiratory difficulty, apparently due to obstruction in the trachea. The animal was etherized, a blood clot was removed from the trachea, a cannula inserted, and the neck bandaged.

The next morning his condition was good and he was walking about, but as considerable pus was coming from the tracheal cannula it was decided to kill him. He was etherized and the carotid artery was again connected with the manometer.

10.30—Blood pressure 106 mm.

11.00—Blood pressure 112 mm.: the difference being due to the depth of anesthesia. The dog was then killed.

Beyond the infection about the trachea, there were no macroscopic lesions found at autopsy.

EXPERIMENT XIX. *Resuscitation five minutes after apparent death.* (Fig. 2.) Mongrel in rather poor condition; weight 7.3 K. Anesthetized with ether.

10.35—Control blood pressure 140 mm.

10.42—Trachea clamped. Several control determinations of blood pressure showing stages of asphyxia were made.

10.44—Last respiration occurred.

10.47—Heart sounds had ceased, and pulse was not palpable.

10.54½—Resuscitation started: Infusion of saline through axillary artery, the infusion bottle being placed at a height of five feet, plus two c.c. solution of adrenalin; artificial respiration was begun at 10.55 without any heart massage. The heart began to beat in about one minute, the blood pressure rising quickly to 206 mm. from which it gradually declined to 130 mm. at 11.10.

11.07—Spontaneous respirations occurred. Artificial respiration was stopped.

11.18—Ether required.

11.50—Control blood pressure 128 mm.

12.05—Control blood pressure 122 mm.

11.15—Control blood pressure 122 mm. Ten mg. of morphine per kilo was administered. Ether was discontinued.

2.05—Control, blood pressure 108 mm.

2.21—Control. The animal was killed as soon as recovery was apparent.

4.41—Control taken. Five hours and forty minutes after resuscitation the dog's condition was excellent, but on account of the septic condition of the operative wounds he was killed.

EXPERIMENT XLVII. *Temporary Resuscitation.* (Fig. 3.) Mongrel weighing about 10 K.

10.20—Anesthesia with ether started.

10.59—Control blood pressure 110 mm.

11.06—Trachea clamped.

11.09¾—Control showing a stage of asphyxia.

11.11¾—Control showing a stage of asphyxia.

11.12—Heart sounds ceased and pulse was not palpable.

11.22—Started resuscitation (10 minutes). Infusion of Ringer's solution, plus 2 c.c. of adrenalin (1—1000) was given through axillary artery, artificial respiration and firm but gentle heart massage were started simultaneously. The heart beat after two minutes, the blood pressure rising to 220 mm. with the typical adrenalin effect. This level was maintained for over three minutes and the decline was gradual.

11.31—Control blood pressure 180 mm.

11.35—Control blood pressure 80 mm. Pulse wave much smaller. Burning of paw evoked no reaction. There were no corneal reflexes.

11.40—Spontaneous respiration occurred. Control.

11.45—Control blood pressure 50 mm. Pulse wave improved.

11.55½—Control blood pressure 60 mm. Artificial respiration stopped. No corneal reflexes, feeble knee jerk in right hind leg.

12.00½—Control. Sharp reaction to paw burning, followed by a secondary reaction with the higher level maintained. Knee jerk appears in left hind leg.

12.07—Control blood pressure 80 mm. Dilating and burning the anus evokes a vasomotor rise, but no respiratory reflex. Corneal reflex appears in right eye. Pupils were extremely dilated after resuscitation, but now are narrowed to some extent. They do not react to light.

12.15—Control blood pressure 50 mm. Pulse wave weaker. Corneal reflex appears in left eye. Respirations have assumed a normal rhythm.

1.06½—Control blood pressure 50 mm. Corneal reflexes improved. In-

competence of sphincters. The animal is entirely anesthetized to pain and completely unconscious. Rigidity of the hind legs appears. Respirations are becoming more labored and quicker.

2.00—Pupils slowly dilating. Left front leg now resists passive motion slightly. Right front leg is entirely relaxed.

2.45—Front legs are also stiffened. Vasomotor reflex is disappearing. Knee jerks cannot be obtained.

3.00—Control blood pressure 50 mm.

3.15—Rapid cardiac failure.

The only finding at autopsy was œdema of the posterior parts of the lungs, which explained the change in the character of the respirations.

Experiment xxxviii. *Failure to resuscitate the higher centres of the brain.* (Fig. 4.) Pug dog weighing 15.9 K.

10.45—Anesthetized with ether, changing at once to chloroform.

11.25—Control blood pressure 110 mm.

11.37 (52 minutes)—Chloroform forced.

11.37¾—Control.

11.38¼—Respiration stopped; cardiac failure followed quickly.

11.39½—No heart sounds.

11.40½—Control. Heart-beats recurred.

11.42—Final cardiac failure.

11.52 (10 minutes)—Started resuscitation. Infusion of Ringer's fluid through axillary artery, plus artificial respiration, was continued for one and a half minutes, and then 1 c.c. of adrenalin was administered. After waiting one and a half minutes, firm, slow massage of the chest was instituted. After a half minute the heart began to beat and the blood pressure rose to 250 mm. from which apex it quickly declined to 80 mm. in about three minutes, the pulse wave growing progressively larger. Drum stopped.

12.07—Control blood pressure 72 mm. This lowering of the pressure occurred synchronously with lowering the dog's feet.

12.11—First spontaneous respiration.

12.13—Control, showing respiratory curve of the blood pressure.

12.17—Control; clot.

12.21—Control blood pressure 90 mm. Artificial respiration stopped.

12.30—Control blood pressure 120 mm. Tracheal cannula out of position.

1.37—Control blood pressure 70 mm.

1.38—Control. Distinct reaction to burning of paw. Corneal reflexes are barely present. No anesthetic required.

2.00—Control blood pressure 84 mm. The dog was in such excellent shape that it was decided to allow him to recover. After the wounds were closed leaving the tracheal cannula in, he was removed from the table.

5.00 (5 hours)—Animal in good shape but unconscious. No ether had been required. Found dead the next morning.

All the organs and tissues presented at autopsy evidence of infection with B. aerogenes capsulatus. There were no clots in the heart and no macroscopical lesions of the viscera other than the post mortem infection noted.

Experiment xlii. *Resuscitation of smaller part of brain after lapse of more time.* (Fig. 5.) Mongrel, 6.9 K., emaciated but healthy looking.

10.15—Anesthetized with chloroform.

11.13—Chloroform forced (anesthesia during one hour).

11.17¾—Heart stopped before respiration according to manometer tracing.

11.19—Heart-beats recurred for about ten seconds.

11.20—Heart-beats recurred a second time, about twelve in all being recorded.

11.20¼—Heart sounds ceased and the pulse was not palpable.

11.40¼ (20 minutes)—Resuscitation started. Infusion of Ringer's fluid through axillary artery, plus 2 c.c. of solution of adrenalin chloride (1—1000), and artificial respiration were started simultaneously. Gentle pressure at intervals on the chest with the feet lowered was also used. After several minutes the axillary cannula pulled out and was refixed in the right carotid.

11.43 (23 minutes)—Resuscitation was again attempted, artificial respiration having been kept up continuously. In about a minute the heart was noticed to be beating. The blood pressure curve rose rather slowly to a height of 110 mm. with a very irregular pulse wave.

11.48—Respiration recurred.

11.49—Control: blood pressure level has fallen to 50 mm.

12.07—Control blood pressure 36 mm. Burning of paw evokes no reaction.

12.20—Blood pressure 50 mm. Pulse wave much improved. Faint corneal reflex in right eye.

2.00—Control blood pressure 54 mm. Respirations have become more normal in type, but the heart rate is distinctly lowered. Burning of paw evokes a very slight rising of blood pressure only. Knee jerks are present in both hind legs. Corneal reflex is present in the right eye, though feebler than normal; it is just perceptible and delayed in the left. Dog appears insensible to touch sensation, but burning anus causes spontaneous movements of the legs. The pupils do not react to light. The right pupil is more dilated than the left.

4.00—Control, showing rise of pressure due to accidental asphyxia.

4.30—Control blood pressure fallen to 28 mm.

Ante-mortem examination was performed. No clot appeared in the heart and the viscera were apparently normal.

EXPERIMENT XLVIII. *Partial resuscitation of a puppy thirty minutes after apparent death.* (Fig. 7.) Skye terrier puppy, three months old, weight 2 K.; anesthetized with ether. A full dose of hirudin—10 c.c. of a .4 of 1% solution—was given to obviate clotting.

2.55—Control blood pressure 104 mm. Chloroform exchanged for ether and forced.

3.03½—Respiration stopped. Control.

3.06—Heart sounds, then pulse, not perceptible.

3.36 (30 minutes)—Began resuscitation. Infusion of Ringer's solution from the height of four feet, plus 2.5 c.c. of 1—1000 adrenalin through carotid artery, with artificial respiration and gentle heart massage, were started simultaneously. Heart began to beat in about two minutes. Infusion was stopped as soon as the blood pressure began to rise decidedly. In about three minutes the blood pressure reached a height of 94 mm., with a regular and rhythmic heart-beat of good strength.

3.41—Drum stopped.

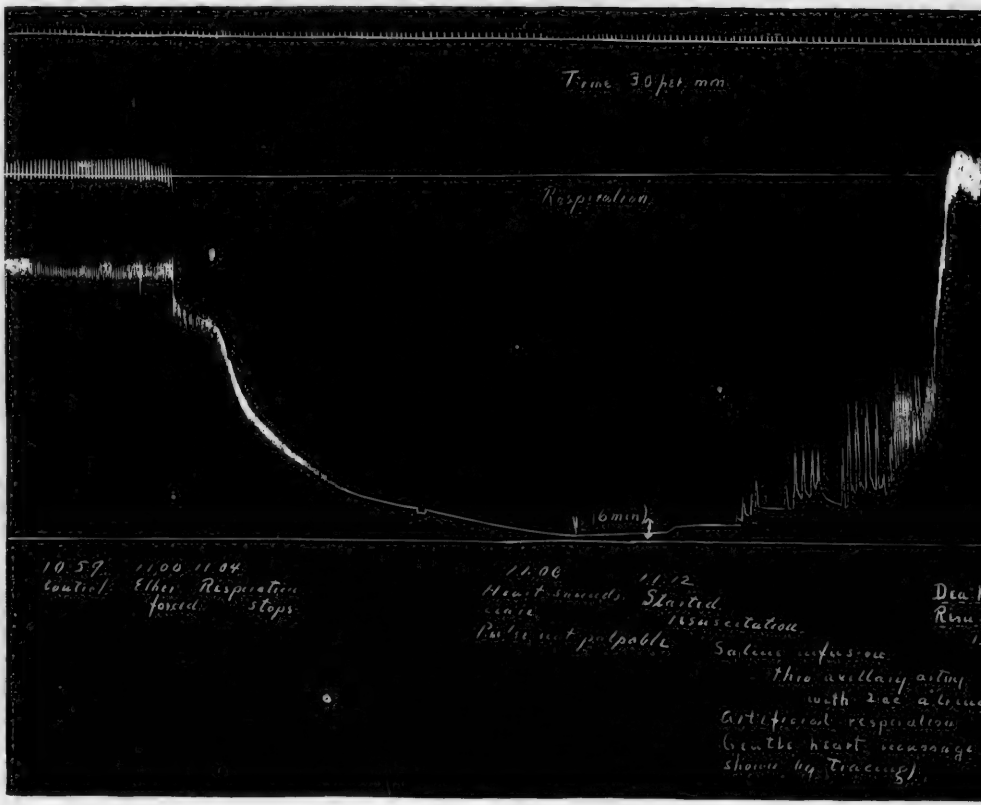
3.50—Control blood pressure 102 mm.

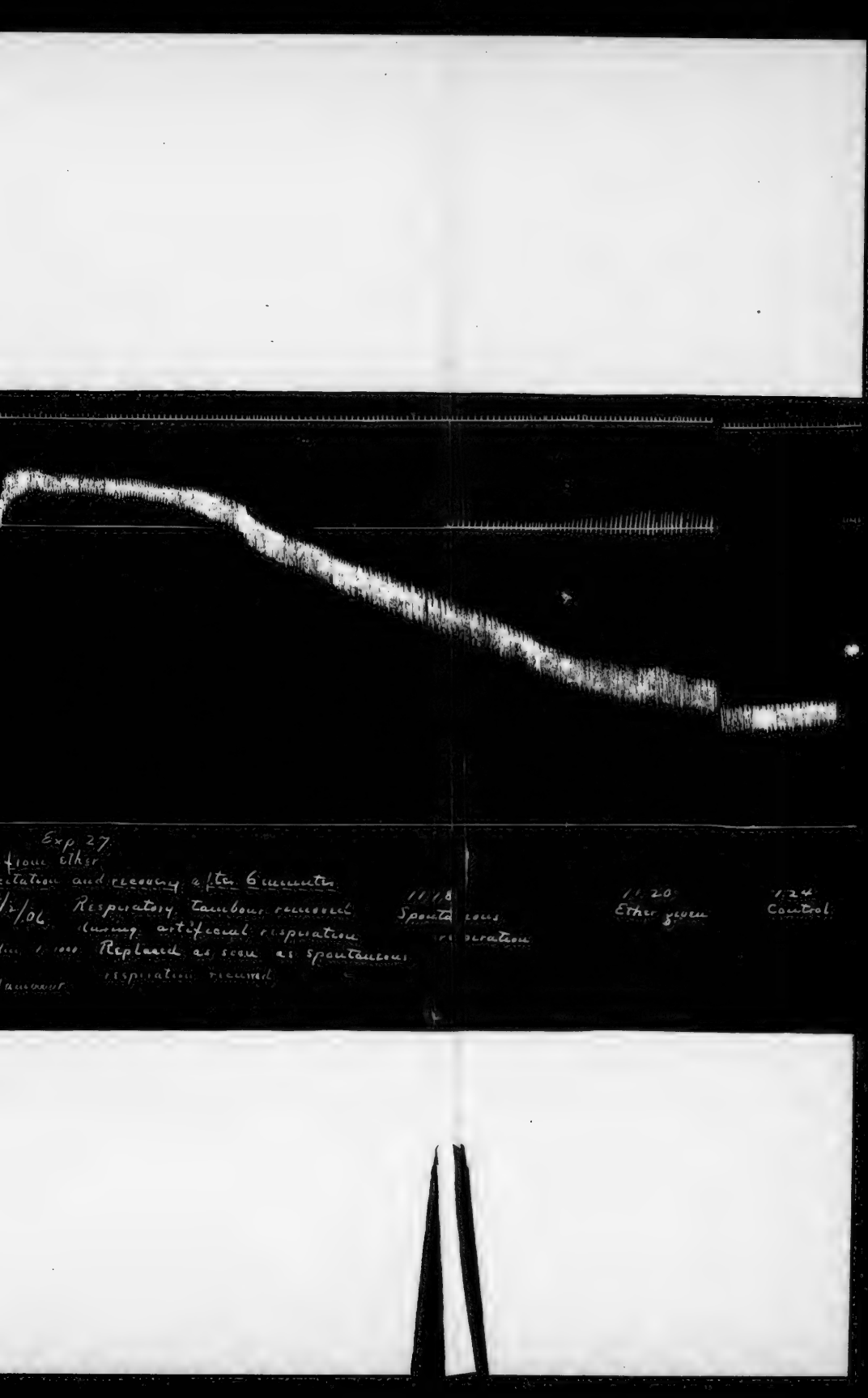
Experiment	Mode of death	Duration of suspended animation (In minutes)		Infusion	Massage	Heart beat: Circulation not maintained	Complete return of circulation.	Length of survival
		Heart	Resp.					
1	Chloroform	2	—	Saline.	Indirect	o	+	½ hr.
2	Asphyxia.	5	—	"	Direct.	+	—	7 min.
3	"	10½	11	"	Vigorous; indirect	+		Short
4	Chloroform	5½	—	"	" "		+	1¾ h. (killed)
5	"	11	21	"	" "		+	10 min.
6	"	6	25	"	" "			12 min.
7	"	21	36	"	" "	+		12 min.
8	"	30	36	"	" "	+		Short
9	"	32	38	"	" "	+		Short
10	Asphyxia.	30	33	"	" "	+		o
11	"	15	19	"	" "	o		o
12	"	15½	—	"	" "	o		o
13	"	11½	16¼	"	" "	o		o
14	Chloroform	6	—	"	Very vigorous		+	3 days (killed)
15	"	17	21	"	Vigorous.	+(?)		?
16	Asphyxia.	11	13	"	"	+		Short
17	"	10½	11½	"	Gentle then direct	+		15 min.
18	Ether	10	17	"	Vigorous.	+		Short
19	Asphyxia	7½	10½	"	o		+	6 hrs. (killed),
20	Ether	7	7½	"	o	o		o
21				o	Vigorous			
22				o	"			
23				o	"			
24	Ether	5	10½	Saline	o			
25	Chloroform	9	13	"	Gentle	o		
26	"	7	12½	"	o	o		
27	Ether	10	—	"	o		+	(killed)
28	"	6	8	"	Gentle		+	24 hrs. (killed)
20	"	15	17	"	Late	o		
30	"	15	24	"	Gentle		+	3½ hrs.
31	Chloroform	7	13	"	"		+	20 hrs.
32	"	6½	—	"	"		—	—
33	"	5	5+	"	After 1 min.	o	—	—
34	"	5	5½	"	After 1 min.	o	—	—
35	"	6½	16½	"	Vigorous	+		Failed at once
36	"	5	10	"	Firm	o		—
37	"	5	8	"	After 2 min.		+	20 hrs.
38	"	7½	9½	Ringer	o	+		Failed at once
39	"	10	13¾	"	Gentle		+	15 hrs. died
40	"	at once	—	Saline	Vigorous		+	15 min. (killed)
41	"	12¾	13	Ringer	Gentle		+	15 hrs. died
42	"	20	21	"	"		+	Short (killed)
43	"	20 (23)	32½	"	At intervals		+	4½ hrs. (died)
44	"	30	33½	"	Gentle	o		—
45	"	30	32½	"	Later vigorous	o		—
46	Asphyxia	18¾	21¼	"	"	o	—	—
47	Ether	25	—	"	"	o		—
48	Asphyxia	10	—	"	Gentle		+	3¾ hrs. (died)
40	Chloroform	30	32½	"	"		+	2½ hrs. (died)
50	"	60	61	"	"		—	—
51	"	45	46	"	"			—
52	"	35	36	"	"	+	+	Short
53	"	40	40½	"	"	+		—
54	"	35	36½	"	"	+		—
55	"	35	36½	"	"		+	Short
56	"	30½	32½	"	o	o		—
57	"	35	36	"	Gentle		+	Short
58	"	7	—	"	"		++	—
59	"	10	—	"	"		++	—
60	"	8	—	"	"		+	—

Summary of Experiments

Length of survival	Respiratory recovery	Vaso-motor recovery	Approximate time of return of heart beat.	Total duration of suspended respiration	Clot	Cardiac dilatation	REMARKS
½ hr.	+	−	½ min.	−	−	−	
7 min.	o	o	6 min.	−	?	−	Window in chest.
Short	o	o	1 min.	−	?	+	Infusion very rapid.
1¾ h. (killed)	o	+	at once	−	?	−	Gréhant anesthesia.
10 min.	+	o	2 min.	−	?	−	Death due to accidental hemorrhage.
12 min.	o	?	2 min.	−	?	+	Rapid infusion.
12 min.	o	o	1 min.	−	?	−	Repeated doses of adrenalin till ineffectual.
Short	o	o	?	−	?	+	Infusion kept up too long.
Short	o	o	Delayed	−	?	−	Effort not uniform nor continuous.
o	o	o	−	−	?	−	Absolute failure.
"	o	o	−	−	?	−	" "
"	o	o	−	−	?	−	" "
o	o	o	−	−	?	−	Delay after starting; auricular appendages beating.
3 days (killed)	+	+	1 min.	−	+	−	Clots at atuopsy in heart.
?	−	o	−	−	+	−	Feeble rythmic beat of right heart at autopsy.
Short	o	o	−	−	+	−	
15 min.	o	o	−	−	+	−	Heart started by direct manipulation.
Short	o	o	Delayed	−	+	−	Massage after failure of first efforts.
6 hrs. (killed),	+	o	1 min.	23 min.	o	−	
o	o	o	−	−	+	o	No massage for 8 min.; auricular appendages beat.
					o		Vigorous massage of beating heart.
					+		Vigorous massage of dying heart.
					o		Vigorous massage with vagus inhibition.
	o	o	−	−	+	+	Vigorous massage after 2½ min.
	o	o	−	−	+	+	Dilatation factor in clotting?
	o	o	−	−	+	+	Clotting without massage.
(killed)	o	o	1 min.	−	o	o	Kitten. Accidental air embolism.
24 hrs. (killed)	+	+	1½ min.	14 min.	o	o	Would have survived.
	o	o	−	−	+	−	Puppy; mass clots.
3½ hrs.	+	Failed	2 + min.	40 min.	o	o	Puppy; hirudin; died.
20 hrs.	+	+	3 min.	22½ min.	o	o	Found dead.
−	o	o	−	−	o	o	Chloroform 1 hour; no artificial respiration.
−	o	o	−	−		+	Rigid chest; old dog.
−	o	o	−	−	+	+	Chloroform 1¼ hours; arythmic beat of auricles
Failed at once	o	o	−	−	+	?	Left in hands of janitor.
−	o	o	−	−	+	+	Clotting in infusion cannula after starting.
20 hrs.	+	+	1 min.	13 min.	o	o	Chloroform ½ hour; delay of 3½ min. after starting.
Failed at once	o	+	1 min.	−	o	o	Chloroform 1 hour; possible over-stimulation of moribund hea
15 hrs. died	+	+	½ min.	32¾ min.	o	o	Chloroform 50 min.
15 min. (killed)	+	−	3 min.	−	o	o	Ether 1½ hours; dilatation overcome by massage and positi
15 hrs. died	+	+	Under 3 m.	23¾ min.	o	o	Chloroform 1 hour; recovered enough to walk.
Short (killed)	−	+	3 + min.	−	o	+	Chloroform 50 minutes; freeing heart started beating.
4½ hrs. (died)	+	+	1 min.	40 min.	o	o	Delay of 3 minutes just at start.
−	o	o	−	−	o	o	Slow infusion.
−	o	o	−	−	+	+	Vigorous massage later.
−	o	o	−	−	+	+	
−	o	o	−	−	+	+	Massage later after first failure.
3¾ hrs. (died)	+	+	2 min.	28 min.	o	o	
2½ hrs. (died)	+	+	2 min.	53½ min.	o	o	Puppy; hirudin; respiration and blood pressure failed.
−	o	o	−	−	o	+	Puppy; hirudin; oedema of lungs.
−	o	o	−	−	o	+	" " "
Short	o	o	3 min.	−	o	+	" " "
−	o	o	−	−	o	+	" " "
−	o	o	−	−	o	+	" " "
Short	o	o	3 + min.	−	o	+	" " "
−	o	o	−	−	o	+	" " "
Short	o	o	5 min.	−	o	+	" no hirudin.
−	+	−	2½ min.	−	o	o	For demonstration.
−	+	−	2 min.	−	o	o	" "
−	+	−	−	−	o	o	" "

UofM


Time 30 per min
Respiration
6 min
10.59
control
11.00 11.04
Ether Respiration
forced stops
11.06
Heart sounds
cease
Pulse not palpable
11.12
Started
resuscitation
Saline infusion
thro axillary artery
Artificial respiration
Gentle heart massage
shown by tracing


Exp 27.
from ether
ciation and recovery after 6 minutes
1/06 Respiratory tambour removed
during artificial respiration
1000 Replaced as soon as spontaneous
respiration resumed
tambour
11.18
Spontaneous
respiration
11.20
Ether given
1.24
Control

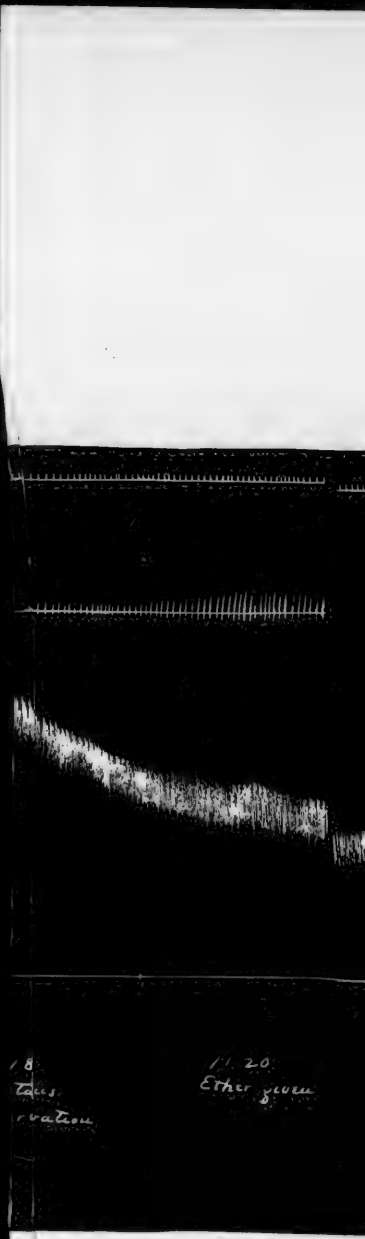
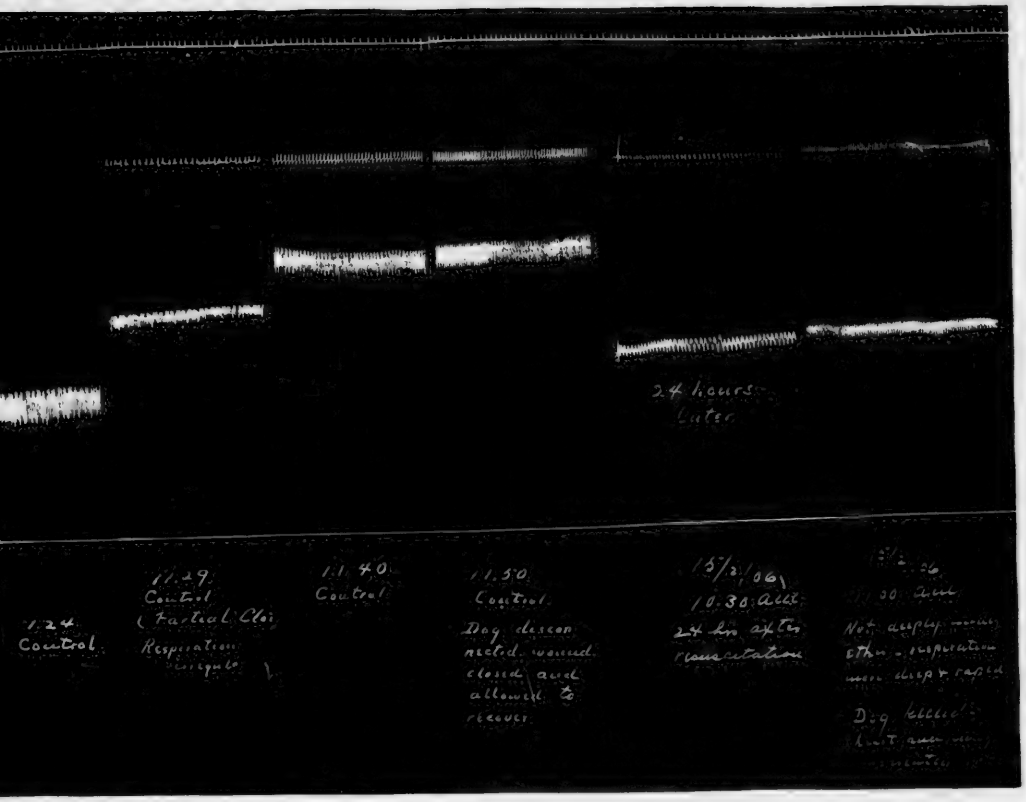
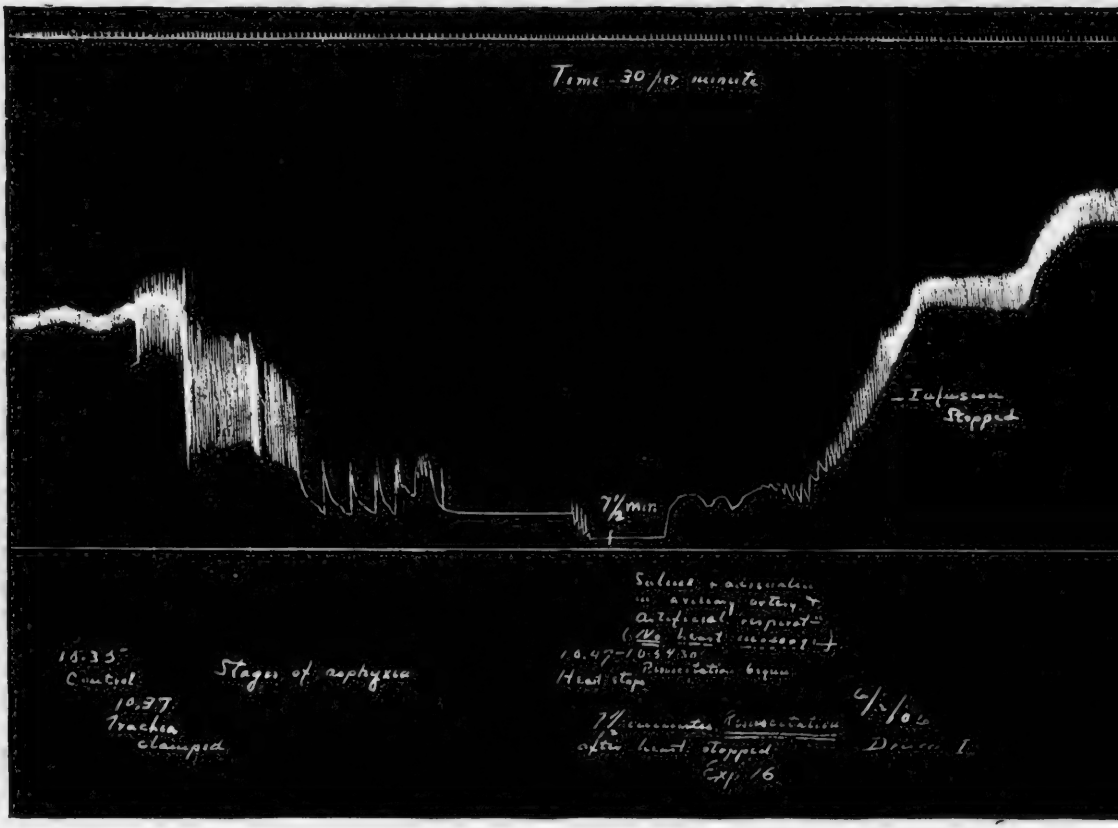

11. 20.
Ether given

PLATE XLII.



Hop N


Time 30 per minute
Infusion Stopped
7½ min
Salines + adrenalin in ascending aorta
Artificial respirat
Heart stopped
10.47–10.49.30
Respiration began
Heart stop
7½ minutes Circulation after heart stopped
Exp 16
10.33
Control
10.37
Trachea clamped
Stages of asphyxia

11.07
Spontaneous
respiration
11.10
Control
11.
Ethe
Drum II
6/2/06

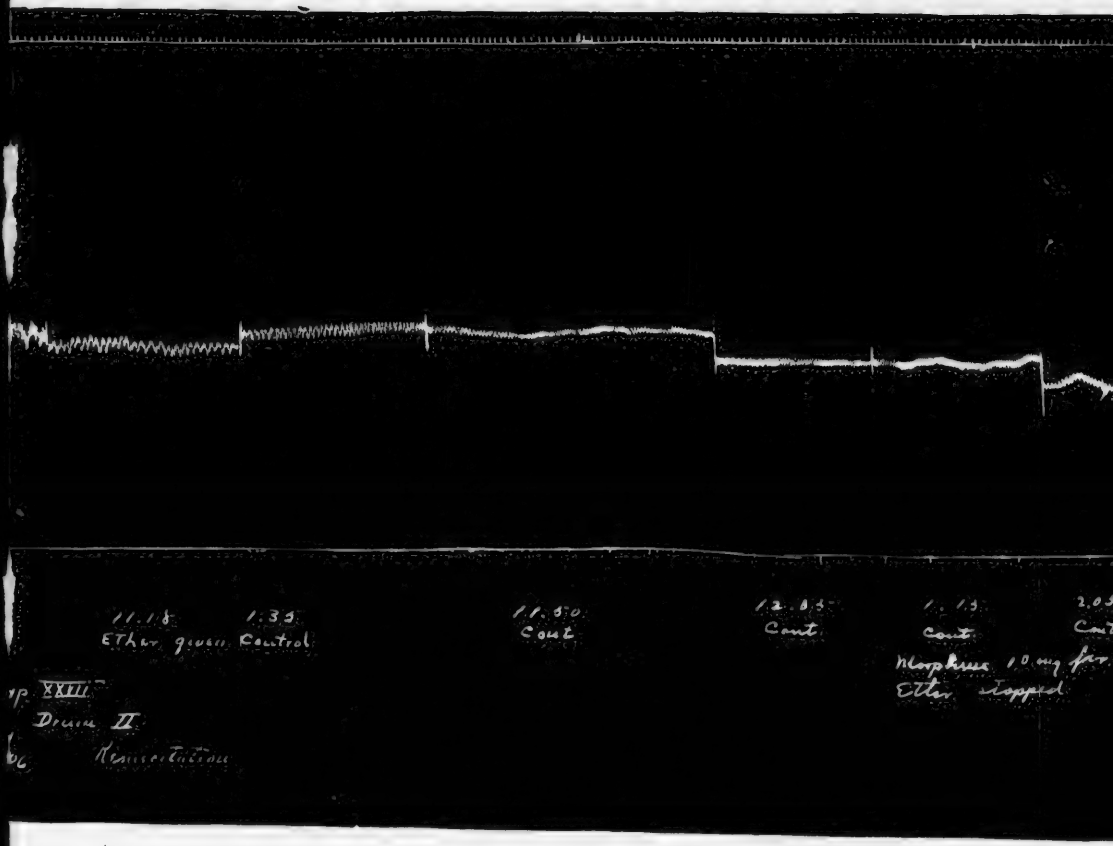
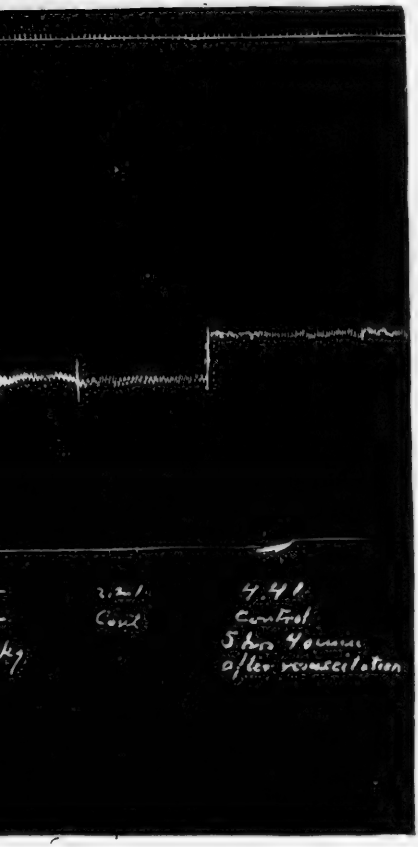
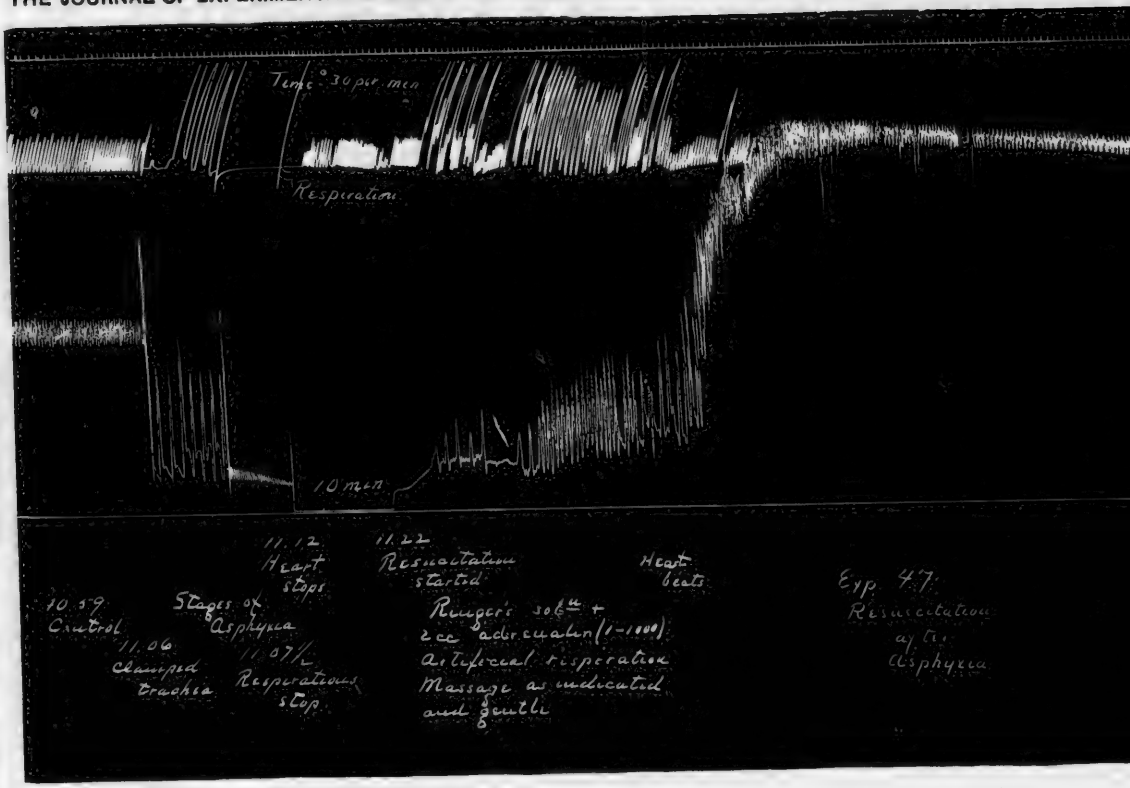
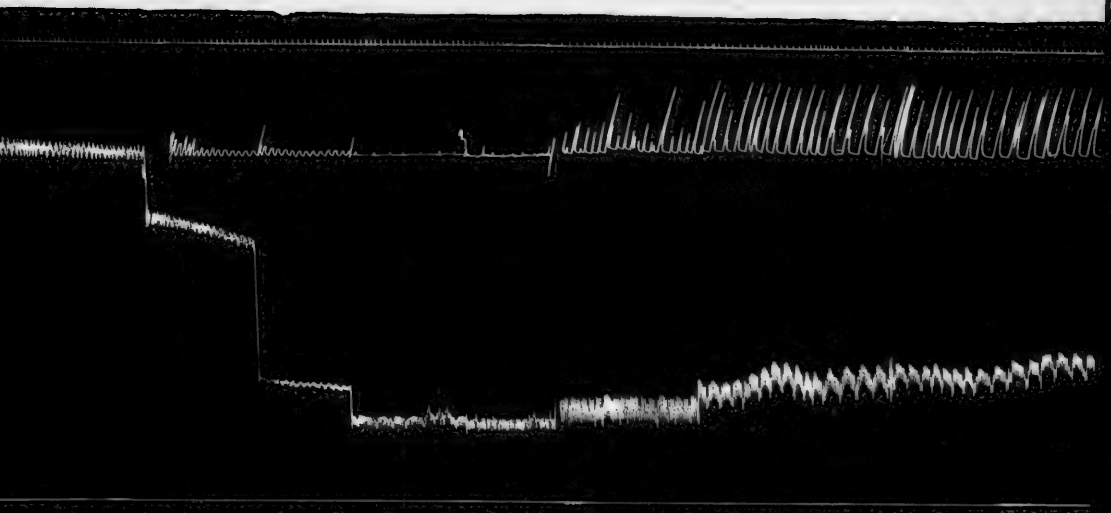
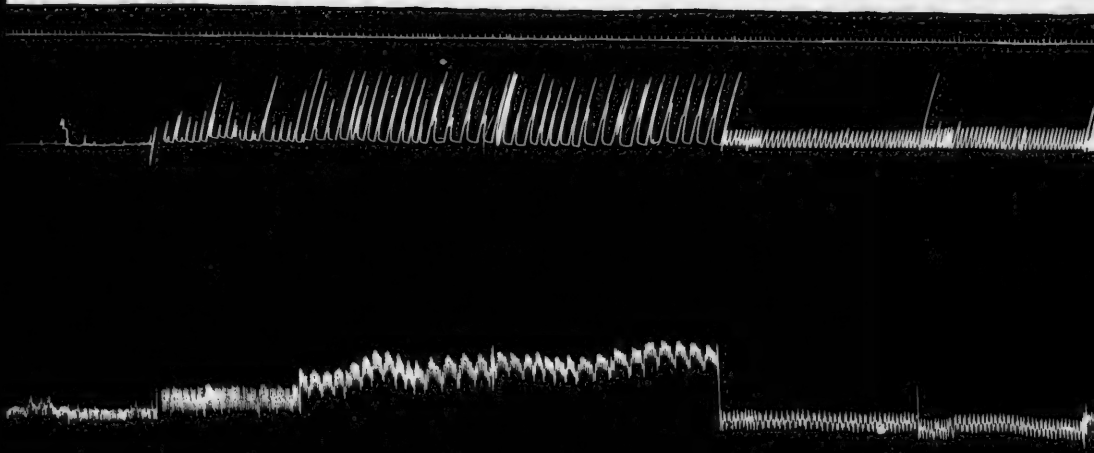

11.18 1.35
Ether given Control
11.50
Cont
12.05
Cont
1.13
Cont
Morphine 1.0 mg
Ether stopped
Exp XXIII
Dr. W. H.
Resuscitation

PLATE XLIII.




Time 30 per min
Respiration
10 min
11.12
Heart stops
11.22
Resuscitation started
Heart beats
Exp. 47.
Resuscitation after asphyxia
10.59
Control
Stages of Asphyxia
11.06
clamped trachea
11.07½
Respirations stop
Ringer's 50cc +
2 cc adrenalin (1-1000)
Artificial respiration
Massage as indicated
and gentle


11.31
Control
11.36
Paw burned
No reaction
11.40
Respiration
returned
11.40
Started
with
respiration
11.43
Paw
burned
11.55½
Control
(Drum II)
12.00½
Paw burned
Knee jerk
appears in
left leg
12.07
Anus burned
Corneal reflex
appears on



1.40 11.45
Started Paw
with burned
respiration

11.55½
Control

(Drum II)

12.00½
Paw burned

Ileus jerk
appears in
left leg

12.07
Arms burned

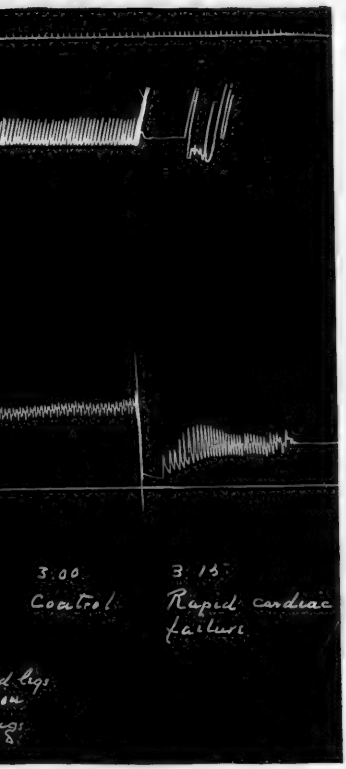
Corneal reflex
appears on rt. eye

12.15
Corneal
reflex
left eye

1.06½
Anesthetic
Incompetence
of sphincter
Rapidity of hu

Alteration in respirat
due to oedema of lu
(autopsy)

PLATE XLIV.



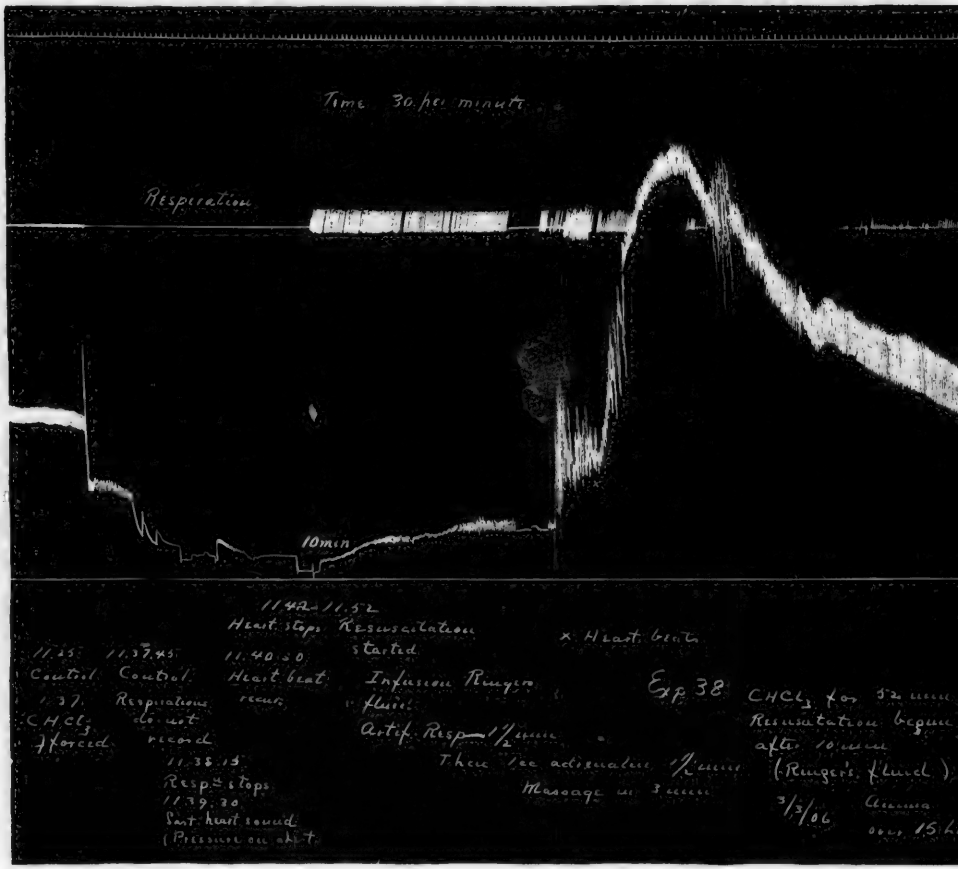
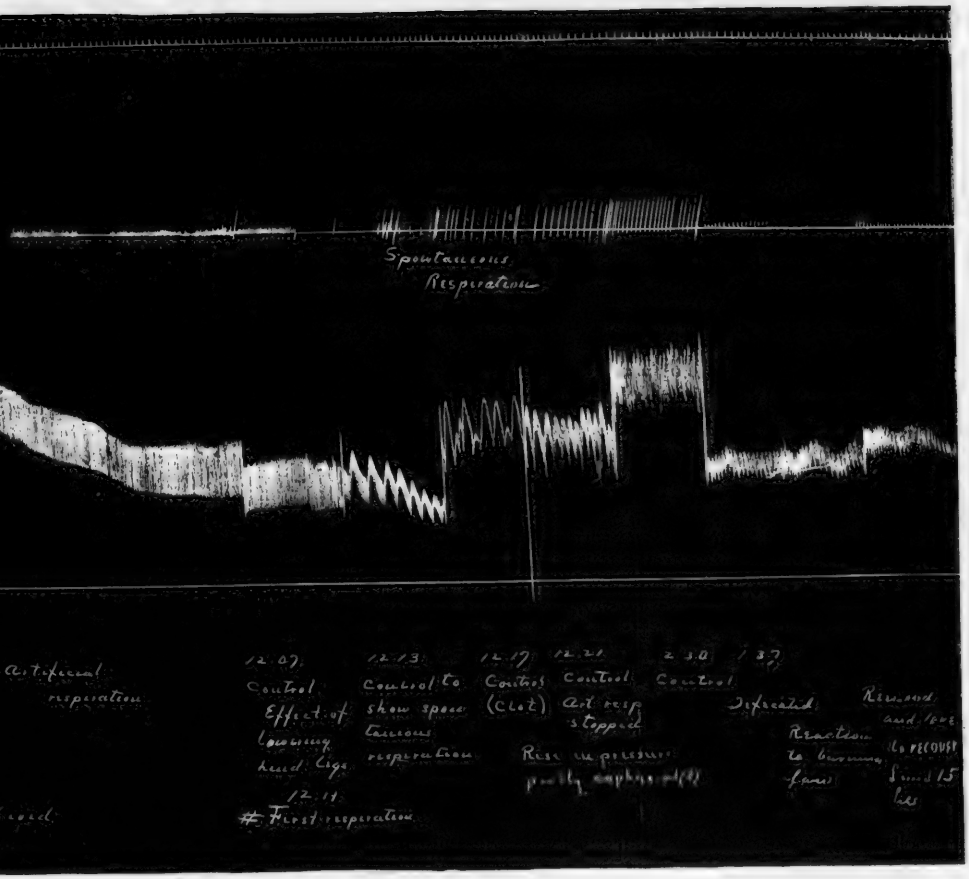
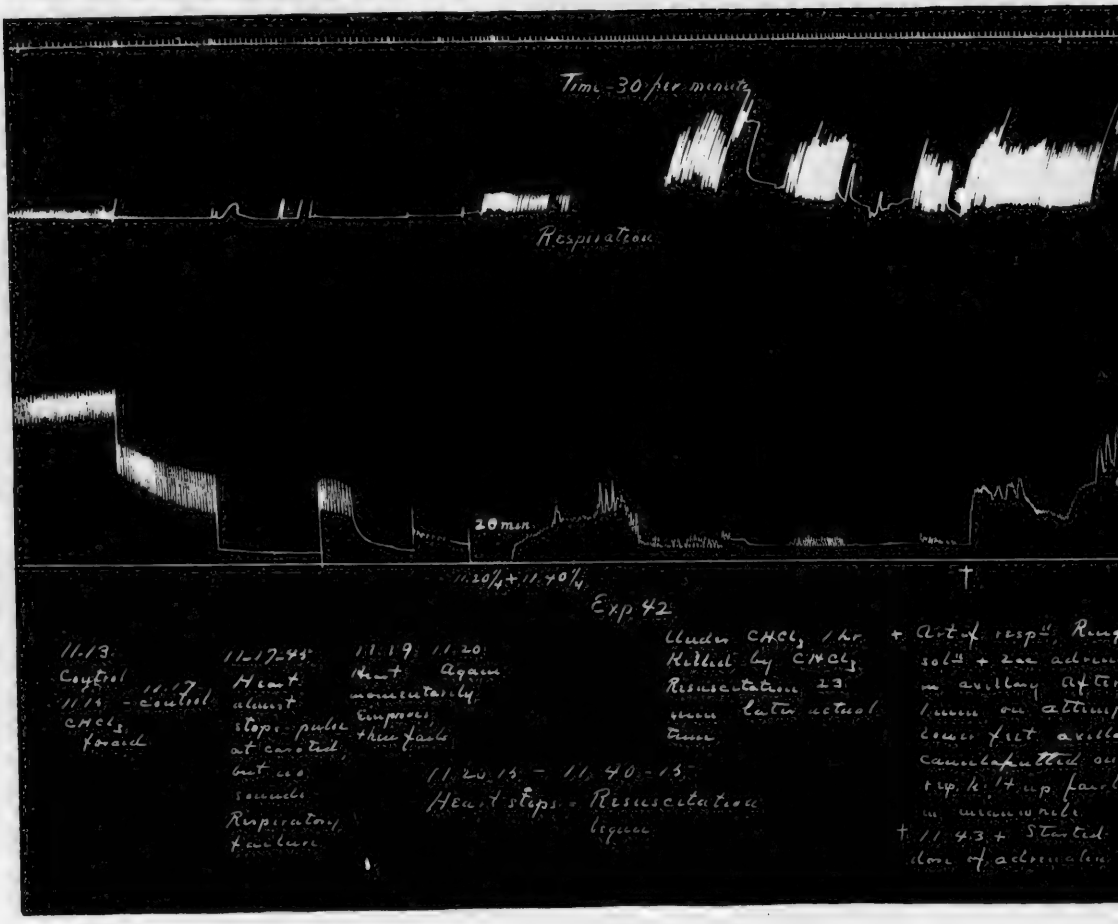
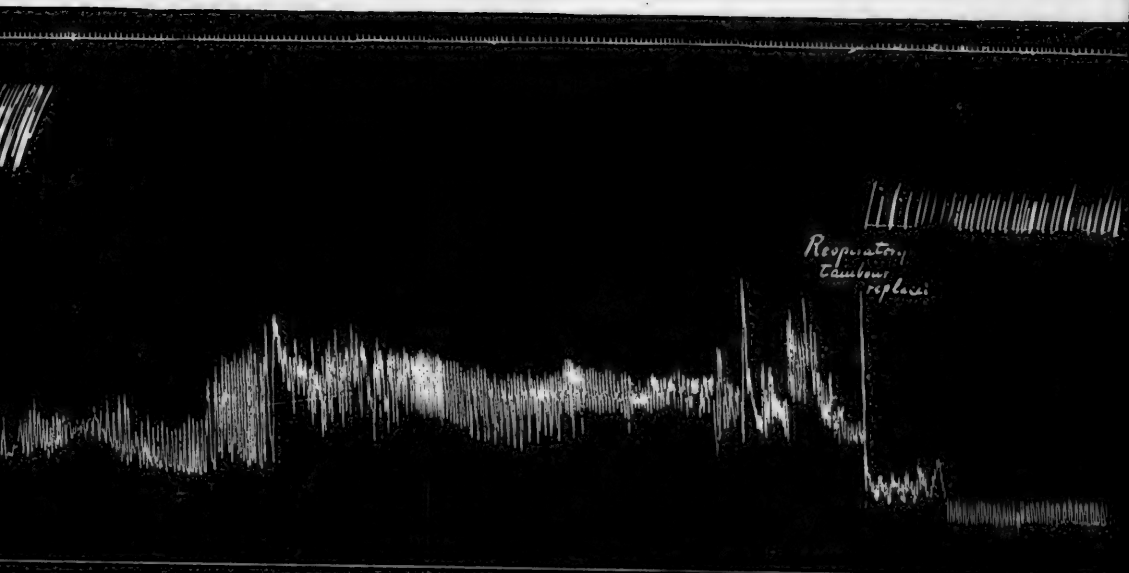

Time 30 per minute
Respiration
10 min
11.42 11.52
Heart stops
Resuscitation started
11.40.30
Heart beat recurs
Infusion Ringer fluid
Artif. Resp 1½ min
There see adrenaline 1/2 mm
Massage 3 mm
11.35 Control
11.37 CHCl3 Forced
11.37.45 Control Respiration do not record
11.38.15 Resp. stops
11.39.20 Last heart sound (Pressure on abt
x Heart beats
Exp 38 CHCl3 for 52 mins
Resuscitation begun after 10 mins (Ringer's fluid)
3/3/06

PLATE XLV.



U of M


Time 30 per minute
Respiration
20 mm
11.20¼ + 11.40¼
Exp 42
11.13 Control 11.17 11.14 - control CHCl3 forced
11.17-45 Heart almost stops pulse at carotid, but no sounds Respiratory failure
11.19 11.20 Heart Again momentarily improves then fails
11.20.15 - 11.40-15 Heart stops - Resuscitation begun
Under CHCl3 1 hr Killed by CHCl3 Resuscitation 23 hours later actual time
+ Art of resp. Resp sol + 2cc adren in axillary Aft 1 hour on attempt lower left axilla cannulated on resp. left up fem in usual way
† 11.43 + Started dose of adrenalin



O Heart noticed to be beating

11.46 Respiration recurs

11.49 Control

Respiratory tambour removed on account massage manipulations

20/ Control Burned paw

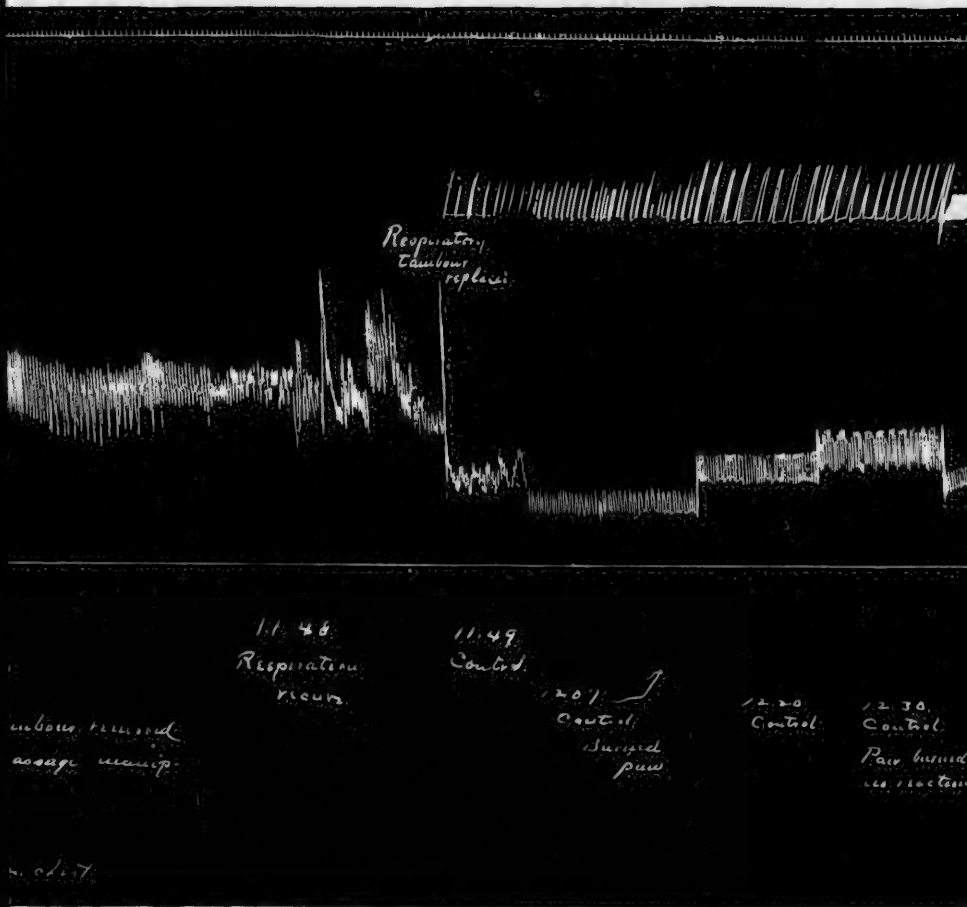
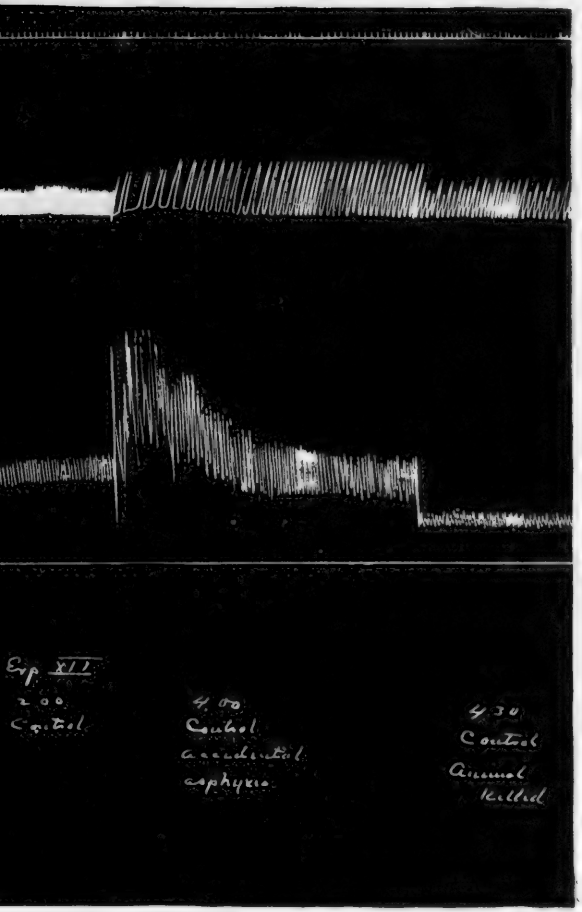

Respiratory tambour replaced
11.46
Respiration recurs
11.49
Control
12.01
Control
Surgical pain
12.20
Control
12.30
Control
Paw burned
on electr

PLATE XLVI.


Exp XLI
2.00
Control
4.00
Alcohol
accidental
asphyxia
4.30
Control
Animal
killed

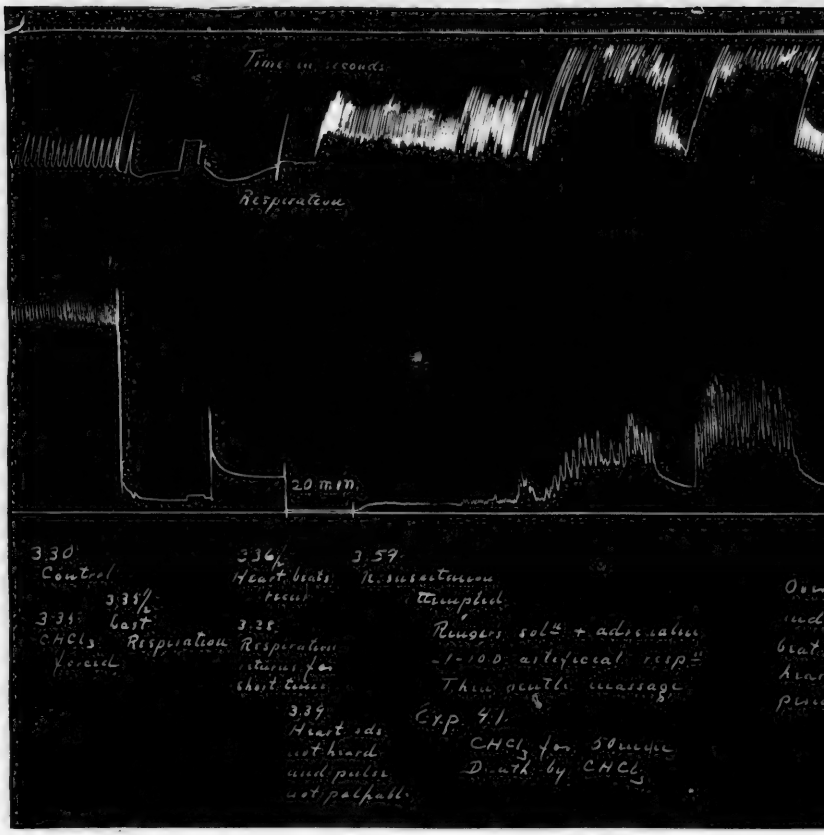
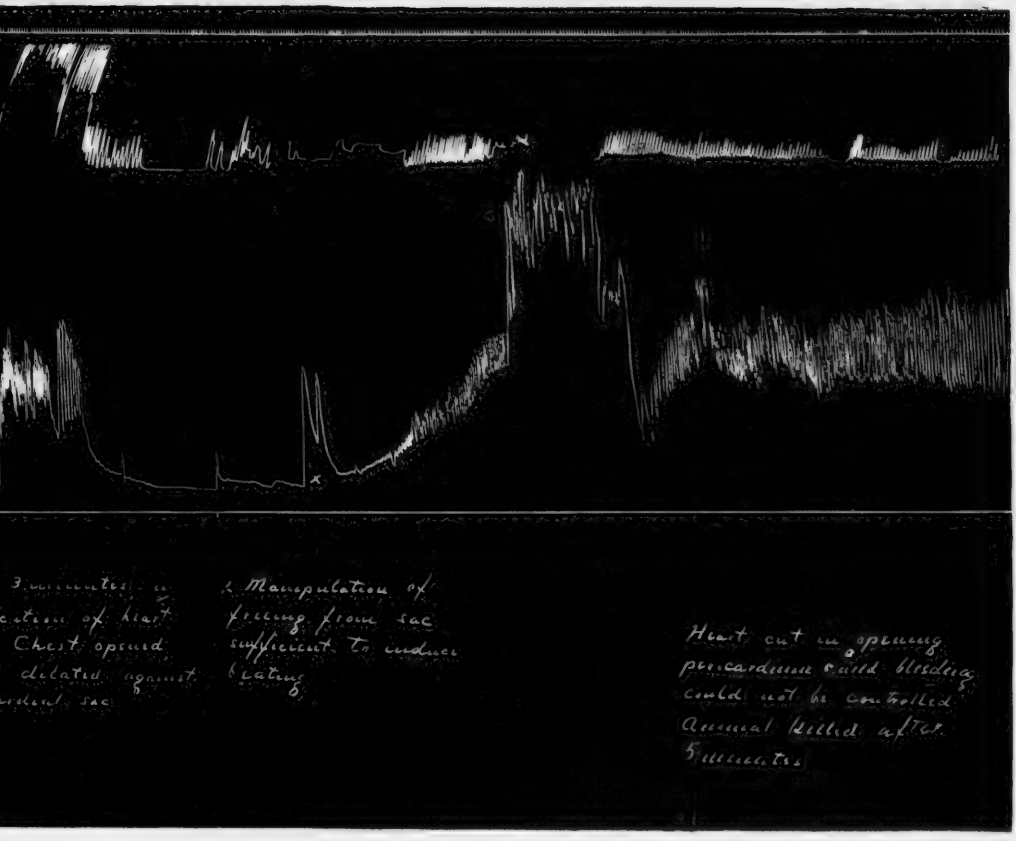

Time in seconds
Respiratory
20 min.
3.30
Control
3.35
3.35½
Last
CHCl₃
Respiration
forced
3.36½
Heart beats
recur
3.28
Respiration
returns for
short time
3.39
Heart sds.
not heard
and pulse
not palpable
3.59
R. subcutaneous
triumphal
Ringer's sol.ⁿ + adrenalin
-1-100 artificial resp.ⁿ
Thor. acutis massage
Exp. 41.
CHCl₃ for 30 minutes
Death by CHCl₃

PLATE XLVII.



3 minutes in
action of heart
Chest opened
dilated against
ardiac sac

2 Manipulation of
heart from sac
sufficient to induce
beating

Heart cut in opening
pericardium & blood
could not be controlled
Animal killed after
5 minutes

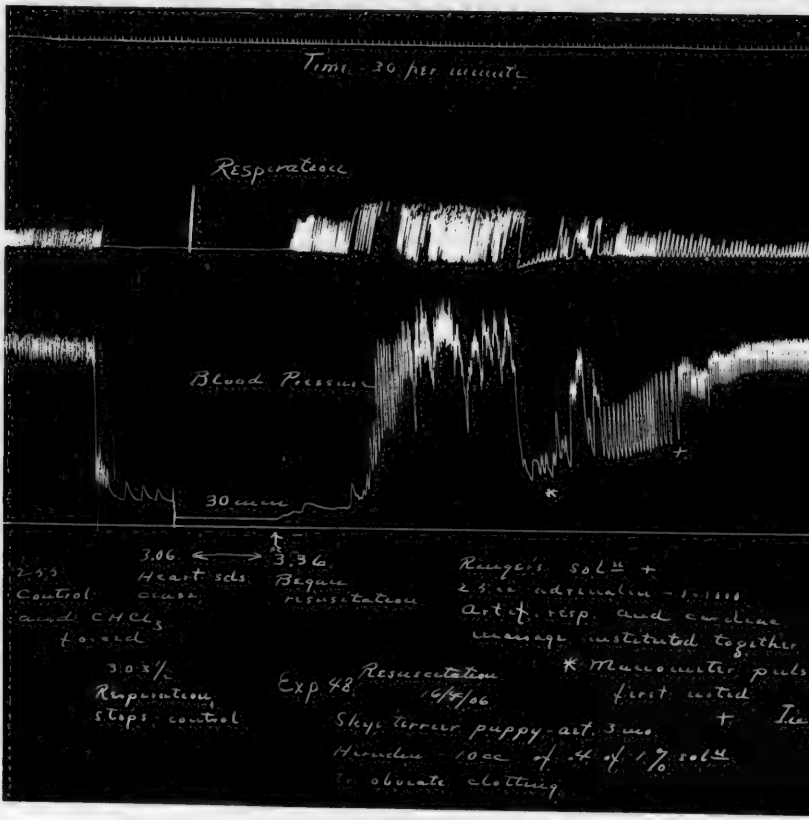
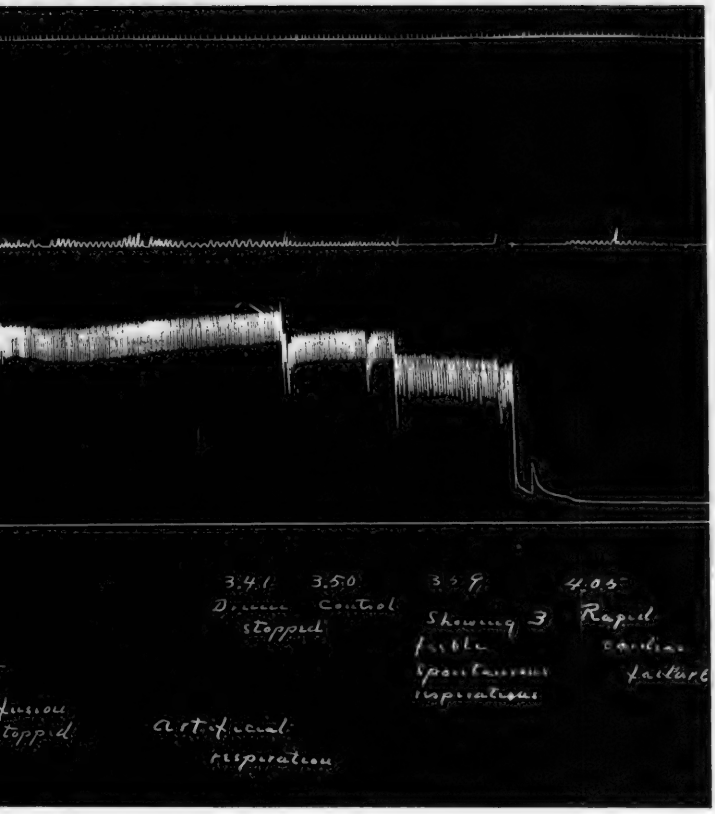

Time - 30 per minute
Respiration
Blood Pressure
30 mm
3.06 ⟷ 3.36
Heart sds. ceases
Begin resuscitation
Ringer's sol.m +
2.5 cc adrenalin - 1:1000
Art. & resp. and cardiac massage instituted together
Control and CHCl3 forced
3.03 Respiration stops
Exp 48 Resuscitation 16/4/06
Skye terrier puppy - art. 3 mo.
Hemolus 10 cc of .4 of 1% sol.m
to obviate clotting
* Manometer pulse first noted
+ Ti

PLATE XLVIII



U of M

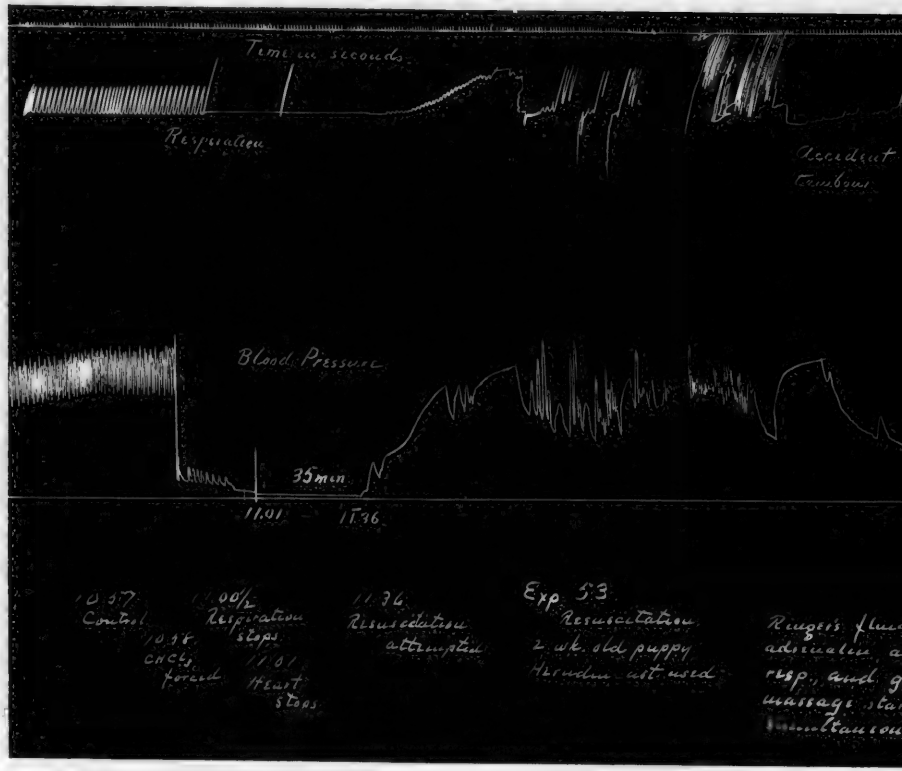
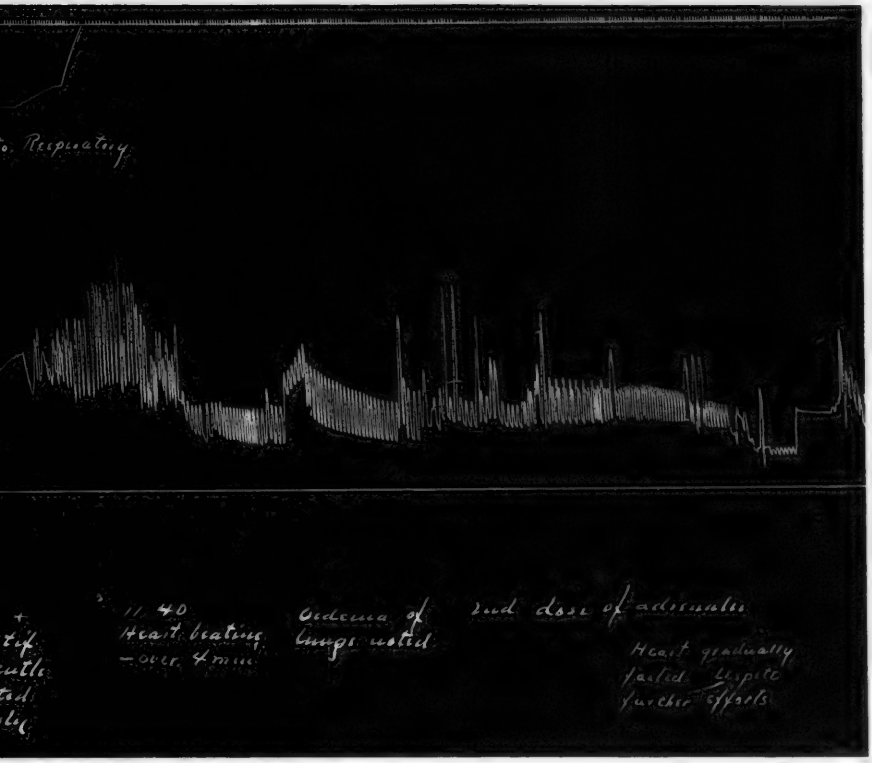

Time in seconds
Respiration
Accident
Blood Pressure
35 min
11.01
11.36
10.57
Control
11.00
Respiration
stops
10.58
cncls
forced
11.01
Heart
stops
11.36
Resuscitation
attempted
Exp. 53
Resuscitation
2 wk. old puppy
Hirudin not used
Ringer's
adrenalin
resp., and
massage

PLATE XLIX.


Respiratory
11.40
Heart beating
—over 4 min.
Oedema of
lungs noted
2nd dose of adrenaline
Heart gradually
failed despite
further efforts

3.59—There are three feeble spontaneous respirations which the control shows. These occurred after several attempts to induce them by stopping artificial respirations, burning anus, and pulling on the tongue. Artificial respiration was continued, but the heart began at once to fail.

4.05—Heart stopped beating entirely.

Complete resuscitation within six minutes has been obtained in a number of experiments since the foregoing was sent to press. Later experiments have apparently established the unequal susceptibility for resuscitation of various parts of the brain.

THE THERMOSTABILE ANTICOMPLEMENTARY CONSTITUENTS OF THE BLOOD.

By HIDEYO NOGUCHI, M.D.,

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(*From the Rockefeller Institute for Medical Research, New York.*)

The present article deals with the antihæmolytic principles of the blood, and especially of normal blood serum, which act against that form of hæmolysis caused by the amboceptor-complement complex.

The present state of our knowledge permits us to divide the antihæmolytic principles of normal blood into two classes. The principles of the first class are directed against certain definite hæmolysins—glucosides, mercuric chloride, agaricin—and certain hæmolytic toxins of micro-organic origin (bacterial toxins). The principles of the second class are directed against the more complex hæmolysins as they exist in normal and immune sera. Preliminary to the consideration of the immediate subject of this paper I will enumerate the factors, other than those which come into play in connection with the normal serum, which influence the process of hæmolysis.

The hæmolytic process can be altered and reduced in different ways. Temperatures above 50° C. render all complements with few exceptions [1],[2] inactive. Divers micro-organisms,[3] organic tissues,[4] certain tissue constituents,[5] and the complex antigen-antibody to which belong the complement fixation of specific precipitates [6-12] all reduce or prevent hæmolysis by interfering under certain conditions with the union of complement and hæmolytic amboceptor. Bordet [13],[14]

[1] Ehrlich and Morgenroth, Ueber Hämolysine, Zweite Mittheil., *Berl. Klin Woch.*, 1899, xxxvi, 481.

[2] Noguchi, On the heat lability of the complements of cold-blooded animals, *Centralbl. f. Bakteriologie*, 1903, xxxiv, 283.

[3] M. Wilde, Ueber die Absorption der Alexine durch abgetötete Bacterien, *Berl. klin. Woch.*, 1901, xxxviii, 878.

[4] V. Dungern, Beiträge zur Immunitätslehre, *Münch. med. Woch.*, 1900, xlvii, 677.

and Ehrlich and Morgenroth [15] describe anticomplements in the strict sense, the existence of which is denied by Moreschi, who accounts for the effect ascribed to them by the complement binding power of specific precipitates.

Ehrlich first described in relation to tetanolysin the antihæmolytic property of normal serum, and Kraus and Clairmont,[16] found this property to be exerted against many bacterial hæmolysins. Neisser and Wechsberg [17] observed the neutralization of staphylolysins by normal guinea-pig serum. A great advance was made by Ransom's [18] discovery of the antihæmolytic action of cholesterin upon saponin. Noguchi,[19] following upon Ransom's discovery, found that cholesterin prevented hæmolysis by agaricin and tetanolysin. These observations drew active attention to the part played by the lipoids of the blood in relation to hæmolysis. P. Th. Müller,[20] Kyes and Sachs,[21] Landsteiner and v. Eisler,[22] Pascucci,[23] studied the nature of the antihæmolytic bodies of normal serum. Müller confirmed the antihæmolytic property of cholesterin. Kyes and Sachs found that cholesterin has no antihæmolytic power against staphylolysin and v. Eisler [24] located the antistaphylolytic principle in the globulin fraction. Pribram [25] found that cholesterin is active against the Nasik vibriolysin; and E. Abderhalden and E. R. Le Count [26] attempted to locate the groups upon which depend the antihæmolytic properties of cholesterin, finding that the free hydroxyl group has a certain relation to these properties. Detre and Sellei [27] stated that lecithin is capable of preventing sublimate hæmolysis.

The investigations summarized indicate that the normal serum contains definite chemical substances which can prevent the hæmolysis caused by bacterial toxins, mineral chemicals, and certain vegetable principles of definite composition. Some differences of opinion exist regarding the nature of the inhibiting substances. Undoubtedly the lipoids of the serum as represented by cholesterin and lecithin possess this property. Apparently a similar property resides in the proteids of the serum. In regard to the latter the question which would at

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[5] P. A. Levene and E. R. Baldwin, On the antihæmolytic action of some cell and tissue constituents, *Jour. of Med. Research*, 1904, xii, 205.

[6] Gengou, Sur les sensibilisatrice des serums actifs contre les substances albuminoides, *Ann. Inst. Pasteur*, 1902, xvi, 734.

[7] F. P. Gay, The fixation of alexines by specific serum percipitates, *Centralbl. f. Bakteriologie*, 1905, xxxix, 603.

[8] Moreschi, Zur Lehre von den Antikomplementen, *Berl. klin. Woch.*, 1905, xlii, 1181.

[9] Neisser and Sachs, Ein Verfahren zum forensischen Nachweise der Herkunft des Blutes, *Berl. klin. Woch.*, 1905, xlii, 1388.

[10] Pfeiffer and Moreschi, Ueber scheinbare Antikomplementär- und Antiamboceptorwirkung precipitirender Sera im Thierkörper, *Berl. klin. Woch.*, 1906, xliii, 33.

[11] Liefmann, Ueber die Komplementablenkung bei Precipitationsvorgängen, *Berl. klin. Woch.*, 1906, xliii, 448.

[12] Muir and Martin, On the deviation of complement by a serum and its antiserum and its relations to the precipitin test, *Jour. of Hygiene*, 1906, vi, 265.

[13] Bordet, Les serums hémolytiques et leurs antitoxines, *Ann. Inst. Pasteur*, 1900, xiv, 257.

once arise is whether all the lipoids had been separated from the proteid fraction of the serum.

A second class of antilytic substances, present in normal serum, are the direct antagonists of complements and amboceptors. Camus and Gley [28] found that heating eel and other normal sera to 56° C. develops in them an antihæmolytic property which they regard as acting antagonistically to the lytic principles. Müller[29] observed this behavior of sera heated to 56° C. and, in addition, the antilytic property exerted by certain unheated sera. Ehrlich and Sachs [30] and Sachs [31, 32] found that at 60° C. but not at 50° C. dog serum becomes anticomplementary. According to their view the zymotoxic group, but not the haptophoric, is destroyed by the higher temperature whereby complementoid is produced. Heating does not, however, leave the haptophore group intact, since complementoid exerts weaker affinity for amboceptor than the original complement. Although dog serum is not rendered anticomplementary when heated to 49° to 50°C., yet it is rendered inactive. No adequate explanation of this inactivity has been advanced. Ox serum was also observed to become anticomplementary when heated to 60° C., but the existence of complementoid could not be demonstrated, because no union between its amboceptor and guinea-pig corpuscles occurs in the absence of normal complement (horse). Recently Bordet and Gay[33] have shown that other factors operate in this phenomenon. Guinea-pig corpuscles can really be sensitized by ox amboceptor, but when the excess of inactive ox serum is removed, horse complement does not exert a dissolving action on the corpuscles (although guinea-pig complement dissolves them promptly). Hence they assert that inactivated ox serum contains bodies which energize the power of horse serum. Neisser and Döring[34] observed in a case of uraemia that the serum, on heating to 56° C., became anticomplementary. Neisser and Friedemann [35] made a similar observation, and in addition they found that heating to 57° C.

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[14] *Idem.* Bemerkung über die Antikomplemente, *Berl. klin. Woch.*, 1906, xliii, 17.

[15] Ehrlich and Morgenroth, Ueber Hämolysine, Vierte Mitth., *Berl. klin. Woch.*, 1900, xxxvii, 681.

[16] Kraus and Clairmont, Ueber Bacteriohämolysine und Antihämolysine *Wien. klin. Woch.*, 1901, xiv, 1016.

[17] Neisser and Wechsberg, Ueber das Staphylotoxin, *Zeitschr. f. Hygiene*, 1901, xxxvi, 299.

[18] Ransom, Saponin und sein Gegengift, *Deut. med. Woch.*, 1901, xxvii, 194.

[19] Noguchi, The antihæmolytic action of blood sera, milk, and cholesterin upon agaricin, saponin, and tetanolysin, *Centralbl. f. Bakteriologie*, 1900, xxxii, 377.

[20] P. Th. Müller, Geht das Tetanolysin mit den Proteiden des Serums und des Eiklares eine ungiftige Verbindung ein? *Centralbl. f. Bakteriologie*, 1903, xxxiv, 567.

[21] Kyes and Sachs, Zur Kenntniss der Cobragift activirenden Substanzen *Berl. klin. Woch.*, 1903, xl, 59, Footnote 3.

[22] Landsteiner and v. Eisler, Ueber Agglutinin-und Lysinwirkung, *Centralbl. f. Bakteriologie*, 1905, xxxix, 309.

[23] Pascucci, Die Zusammensetzung der Blutscheibenstromes und die Hæmilyse, *Hofmeister's Beiträge*, 1905, vi, 552.

inactivated the serum without destroying the anticomplementary action. They endeavored to account for the phenomenon by supposing the amboceptor of uraemic blood to be less heat stabile than normal, whereby, at 56° C., the cytophylic group is destroyed and the complementary one is left intact (amboceptoid) and capable of deviating complement. They thought to prove their view by pointing out that extraction of the heated serum by susceptible red corpuscles did not remove the inhibitory action of the serum. Unfortunately, they seem not to have tested the corpuscles for resistance. Laqueur[36] confirmed the work of these investigators and found, in addition, not only that similar conditions existed in the blood in other pathological states, but that they may be absent from uraemic blood. Senator[37] failed to find the phenomenon in classical examples of uraemia, and Bergmann and Keuthe[38] ascertained that it is irregular in its occurrence in uraemia and occurs in other pathological conditions. That rabbit serum may, after heating to 56° C., develop anticomplementary action is shown by certain experiments of Sachs[39] and Pfeiffer and Friedberger.[40] Muir and Browning[41] mention that guinea-pig serum, from which complement has been removed by means of sensitized corpuscular stromata, previously heated to 56° C. over night and washed, does not become anticomplementary upon heating to 55° C., because no complement exists to be changed into complementoid. Manwaring[42] describes a qualitative change in heated immune serum after digestion with corpuscles, but does not define accurately this alteration.

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[24] v. Eisler, Bedeutung der Lipoide für die antihämolytische Wirkung des Blutes, *Zeitschr. f. exper. Pathologie und Therapie*, 1906, iii, 296.

[25] Pribram, Ueber Bakteriohämotoxine (Lysine) und Antihämotoxine, Handbuch der path. Mikroorganismen, 1906, Ergänzungsband, i, 291.

[26] E. Abderhalden and E. R. Le Count, Die Beziehungen zwischen Cholesterin, Lecithin, and Cobragift, Tetanustoxin, Saponin und Solanin, *Zeitschr. f. exper. Pathologie und Therapie*, 1905, ii.

[27] Detre and Sellei, Hämolytische Wirkung des Sublimates, *Wien. klin. Woch.*, 1904, xvii, 1195, 1234.

[28] Camus and Gley, A propos de l'existence, dans un serum sanguin, d'une action antagoniste de l'action hémolytique, *Compt. rend. d l. Soc. d. Biologie*, 1901, liii, 732.

[29] P. Th. Müller, Ueber die Antihämolysine normaler Sera, *Centralbl f. Bakteriologie*, 1901, xxix, 860.

[30] Ehrlich and Sachs, Ueber den Mechanismus der Amboceptorenwirkung *Berl. klin. Woch.*, 1902, xxxix, 492.

[31] H. Sachs, Giebt es einheitliche Alexinwirkung? *Berl. klin. Woch.*, 1902, xxxix, 216.

[32] *Idem*, Ueber Komplementoide, *Centralbl. f. Bakteriologie*, 1905, xl, 125.

[33] Bordet and Gay, Sur les relations des sensibilisatrice avec l'alexine, *Ann Inst. Pasteur*, 1906, xx, 467.

[34] Neisser and Döring, Zur Kenntnis der hämolytischen Eigenschafte des menschlichen Serums, *Berl. klin. Woch.*, 1901, xxxviii, 593.

[35] Neisser and Friedemann, Ueber Amboceptoidbildung in einem urämischen Serum, *Berl. klin. Woch.*, 1902, xxxix, 677.

The difference in the heat-effects, according as the temperatures to which the sera are exposed are about 50° C. or between 56° and 60° C., is remarkable. If we assume, as Ehrlich and Sachs have done, that complementoid is formed at 60°C., what are we to infer happens at 50° C.? If the anticomplementary effect is caused by destruction of the zymotoxic groups, what changes are produced at 50° C. leading to inactivation without developing any antagonistic activity? The view that the difference depends on the thermolability of the amboceptor is hypothetical, and the experiments adduced by Neisser and Friedemann to uphold it are unconvincing. I shall return to this point later in connection with my experiments which seem to me to afford a solution of the problem.

That the blood lipoids may antagonize hæmolysis by amboceptor-complements has also been shown. Landsteiner and v. Eisler[43] extracted lipoids from blood corpuscles and noted that they exerted an antihæmolytic action against serum hæmolysins. Later, these authors[44] found that blood serum contained similar bodies in smaller amounts than the corpuscles. They believed the action of the lipoids to be anti-amboceptoric, in the sense that they constitute the receptors, which in the intact corpuscles anchor the amboceptors. Bodies having the same action can be obtained from the stroma of corpuscles. Bang and Forssmann[45,46] extracted ox corpuscles with ether and obtained a mixture which, according to them, contains the hæmolysin or amboceptor-producing agent and antihæmolysin (complement-neutralizer). By treating the ethereal extract with aceton they obtained in solution the latter principle; the insoluble residue retained this amboceptor-producing body. This can be separated from phoshatides and cerebrosides by extraction with alcohol, ether, chloroform or cold benzol. Bang and Forssmann regard the amboceptor-fixing body (receptor in Ehrlich's sense) and amboceptor-producing substance as distinct from each other. Landsteiner and v. Eisler and Bang and Forssmann ob-

[36] Laquer, Zur Kenntnis urämischer Zustände, *Deut. med. Woch.*, 1901, xxvii, 744.

[37] H. Senator, Ueber die hämolytische Eigenschaft des Blutserums bei Urämie, *Berl. klin. Woch.*, 1904, xli, 181.

[38] Bergmann and Keuthe, Die Hemmung der Hämolyse durch inactivirte menschliche Sera, *Zeitschr. f. exper. Pathologie und Therapie*, 1906, iii, 255.

[39] H. Sachs, Ueber das Zusammenwirken normaler und immunisatorisch erzeugter Amboceptoren bei der Hämolyse, *Deut. med. Woch.*, 1905, xxxi, 705.

[40] Pfeiffer and Friedberger, Beitrag zur Lehre von den antagonistischen Serumfunktionen, *Centralbl. f. Bakteriologie*, 1906, xli, 223.

[41] Muir and Browning, On the properties of anti-immune bodies and complementoids, *Jour. of Hygiene*, 1906, vi, 1.

[42] Manwaring, The absorption of hemolytic amboceptor, *Jour. of Infect. Diseases*, 1905, ii, 485.

served that stromata of red corpuscles, freed of fat by solvents, are no longer able to fix amboceptor.

I wish, in this place, to point out that the anticomplementary body of Bang and Forssmann is, according to their view, of specific nature. It is questionable, indeed, whether their experiments wholly support this view (their experiments *l. c.* p. 261 can be interpreted so as to show the effect to be upon the amboceptor rather than upon the complement). This body is thermostable: boiling for four minutes does not destroy it, but longer boiling does. It is of some interest to learn that these authors look upon this body as fixing the complement to the corpuscles after it has been exposed for action by the union of corpuscles and amboceptors. Hæmolysis results, therefore, from union of amboceptor and complement separately with the corpuscles.

A review of the literature upon the inhibition of serum hæmolysis brings out from a large mass of hypothesis a few definite facts. It is established that many normal and pathological bloods contain certain constituents which possess the power to suppress the action of serum-complements. This anticomplementary action may be present in the native serum or it may manifest itself only after the serum has been heated to 56° C. or higher. It has been shown that by extraction with certain solvents red corpuscles and serum yield substances which exert antilytic action. Whether the antilytic action of fresh and heated sera depend wholly, or at all, upon these substances is not proven beyond doubt. It is not proven that any of these antilytic substances are merely modified complements and amboceptors, and the existence of complementoid and amboceptoid has not been established. Apparently there are differences of considerable magnitude between the influences of heat and chemicals upon the antilytic action of sera and these extractives. It may be an

[43] Landsteiner and v. Eisler, Ueber die Wirkungsweise hämolytischer Sera, *Wien. klin. Woch.*, 1904, xxvii, 676.

[44] *Idem*, Ueber Agglutinin- und Lysinwirkung, *Centralbl. f. Bakteriologie*, 1905, xxxix, 309.

[45] Bang and Forssmann, Untersuchung über die Hämolysinbildung, Vorl Mitth., *Centralbl. f. Bakteriologie*, 1905, xl, 150.

[46] *Idem*, Untersuchung über die Hämolysinbildung, *Hofmeister's Beiträge*. 1906, iii, 238.

accidental circumstance that the temperatures which destroy complement and amboceptor also remove this antilytic property from the serum. The influence of temperature upon combination and dissociation of serum constituents is probably very wide. The different effects produced at 50° C. are cases in point. Manwaring[47,48] found that normal goat serum, heated to 56° C. to 59°, acquired certain properties which he ascribed to a third serum component. The heated serum might be purely auxilytic, or purely antilytic, or again, auxilytic in certain amounts and antilytic in others, or, even as a fourth possibility, completely inactive. Sera of different normal animals heated under these conditions might differ widely in the action of the third components. No explanation was offered for these variable effects. Possibly this effect is brought about by liberation of certain hæmolytic lipoids from inactive combinations, and these may be powerful enough to overcome the antilytic action of the antilysins whose action we are considering. I have observed many instances of the auxilytic effect described by Manwaring, but sera from which the lipoids have been extracted by alcohol and ether do not develop, on heating, auxilytic properties.

NORMAL SERUM AS ANTIHÆMOLYSIN.

To bring out the antihæmolytic action of normal serum it is customary to inactivate it at 56° C. Either the heated serum and active hæmolytic serum are first mixed and the blood corpuscles added later, or, the heated serum and complement are first brought together and at a later stage the inactive hæmolytic serum (amboceptor) is added. Fresh serum can be tested accurately for antilytic action only when it does not contain suitable complement.

Table I shows the normal sera of the dog and sheep, heated to 56° C., to become antihæmolytic, while the same sera in the fresh state are non-protective. On the other hand, fresh ox serum protects its corpuscles, but heating to 56° C., removes the protective property. Inactivated ox serum is, however,

[47] Manwaring, On auxilysins, *Jour. of Infect. Diseases*, 1906, iii, 225.

[48] *Idem*, The third serum component, *Jour. of Infect. Diseases*, 1906, iii, 645.

TABLE I.

THE ANTIHÆMOLYTIC POWER OF DOG, SHEEP, AND OX SERA WAS TESTED AGAINST THE ACTIVE ANTI-DOG (RABBIT), ANTI-SHEEP (GOAT), AND ANTI-OX (RABBIT) SERA. THE HÆMOLYTIC VALUES OF THESE IMMUNE SERA WERE, IN THE FRESH STATE, 0.05 C.C. FOR ANTI-DOG, 0.07 C.C. FOR ANTI-SHEEP AND 0.05 C.C. FOR ANTI-OX SERUM, UPON THEIR CORRESPONDING CORPUSCLES.

Doses of the normal serum mixed with 0.2 of active immune serum.	Anti-dog serum 0.2						Anti-sheep serum 0.2						Anti-ox Serum 0.2					
	Dog serum		Sheep serum		Ox serum		Dog serum		Sheep serum		Ox serum		Dog serum		Sheep serum		Ox serum	
	Fresh	56° C	Fresh	56° C	Fresh	56° C	Fresh	56° C	Fresh	56° C	Fresh	56° C	Fresh	56° C	Fresh	56° C	Fresh	56° C
1.	CH	none	CH	none	CH	none	CH	none	CH	none	almost CH	none	CH	none	slight	none	much	CH
0.7	"	"	"	trace	"	slight	"	none	"	"	"	slight	"	none	"	"	"	"
0.5	"	trace	"	much	"	almost CH	"	slight moderate	"	trace	"	much	"	slight	"	trace	CH	"
0.3	"	"	"	"	"	"	"	much	"	"	"	"	"	"	almost CH	slight	"	"
0.2	"	"	"	almost CH	"	"	"	"	"	"	"	almost	"	mod'te	CH	mod'te	"	"
0.1	"	"	"	"	"	CH	"	almost	"	slight mod'te	"	CH	"	much almost	"	much almost	"	"
0.07	"	much	"	"	"	"	"	CH	"	much	"	CH	"	CH	"	CH	"	
0.05	"	"	"	CH	"	"	"	"	"	CH	"	CH	"	CH	"	CH	"	CH
0.03	"	CH	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	
0 (control)	CH	CH	CH	CH	CH	CH	CH	CH	CH	CH	CH	CH	CH	CH	CH	CH	CH	

protective for alien corpuscles. The heated sera are in general antihæmolytic for many kinds of corpuscles, against several, at least, different kinds of actively hæmolytic sera. Table II brings out this fact, and it also shows that heated dog serum neutralizes the action of fresh dog serum. There is, therefore, no indication of a specific antihæmolytic action of the heated sera.

TABLE II.

Amount of heated normal serum added to 0.5 c.c. of fresh active dog serum	Sheep corpuscles	Ox corpuscles
Sheep serum (56° C) 1 c.c.	no hæmolysis	slight hæmolysis
Ox serum (56° C) 1 c.c.	moderate hæmolysis	moderate hæmolysis (in other instances none or complete).
Dog serum (56° C) 1 c.c.	moderate "	moderate hæmolysis.
0.9% saline solution 1 c.c.	complete "	complete "

NATURE OF ANTIHÆMOLYTIC ACTION.

It is necessary to decide whether the antagonistic action is directed against complement or amboceptor. A hæmolytic combination of inactivated anti-sheep serum (from goat) and guinea-pig complement was prepared. An amount of 0.1 cubic centimeter of the former and 0.01 cubic centimeter of the latter caused prompt lysis of the sheep corpuscles. Now, to one cubic centimeter of each of the inactivated normal sera 0.01 cubic centimeter guinea-pig complement was added, the mixture being kept at 37° C. for one hour. The sheep corpuscles were then added, and after another half hour, 0.1 cubic centimeter of inactive anti-sheep serum. Reading was made at the expiration of twenty hours; the result was as follows:

Inactivated normal sheep serum (56° C.) 1 c.c. =No hæmolysis.
" " ox " " 1 c.c. = " "
" " dog " " 1 c.c. = " "
Control (no normal serum) saline 1 c.c. =Complete hæmolysis.

The corpuscles were now separated from the different mixtures, resuspended in two cubic centimeters of 0.9 per cent. saline

solution, and 0.1 cubic centimeter guinea-pig complement added to each. The effect was as follows:

Corpuscles treated with inactive sheep serum						= Marked hæmolysis
"	"	"	"	ox	"	= " "
"	"	"	"	dog	"	= Complete hæmolysis

To each of the separated fluids 0.4 cubic centimeter amboceptor for sheep corpuscles was added; no hæmolysis was produced by these mixtures showing no active complement to be present. Hence it is concluded that the antihæmolytic action is due to neutralization of complement.

It may be stated, in this place, that the corpuscles separated from the inactive sera do not undergo lysis as readily as the controls without serum. The latter underwent lysis with 0.01 to 0.02 cubic centimeter guinea-pig serum, while the former required 0.1 cubic centimeter, which amount produced incomplete lysis in some cases. This result might be taken to indicate that the action is anti-amboceptoric, a conclusion which later experiments disproved. Mere digestion of corpuscles in inactive sera raises the resistance to lysis of the corpuscles. The anti-complementary action of the heated sera is also brought out by the next experiments.

Anti-sheep amboceptor (goat) heated to 56° C.	0.4. c.c.
Dog serum heated to 70°	0.5 c.c.

Kept one hour at 37° C., after which were added sheep corpuscles and 0.02 c.c. guinea-pig serum. Result: Complete hæmolysis.

Guinea-pig serum	0.2 c.c.
Dog serum heated to 70° C.	0.5 c.c.

Kept one hour at 37° C., after which sheep corpuscles and finally anti-sheep amboceptor 0.4 c.c. were added. Result: No hæmolysis.

INACTIVATION AND ANTILYSIN OF NORMAL SERA.

That temperatures approaching 60° C. rendered the majority of normal sera antihæmolytic, while somewhat lower temperatures (49° to 50° C.) merely inactivated them, has been established by Müller, Ehrlich and Sachs, Neisser and Friedemann, and others.[49] The changes in the serum through which an antilytic property is developed is considered by Ehrlich and Sachs to be

[49] *Op. cit.*

due to the formation of complementoid and by Neisser and Friedemann of amboceptoid. My own experiments have shown heated sera to be anticomplementary. The next experiments refer to the temperatures at which the antilysin develops.

Normal sheep, dog, and ox sera were tested against anti-sheep, anti-dog, and anti-ox sera. The anti-sera were prepared in rabbits. The anti-sera were inactivated at 50° C. and reactivated with guinea-pig serum. The titres of the anti-sera were about equal; 0.1 cubic centimeter anti-sera and 0.02 cubic centimeter guinea-pig complement was needed for complete hæmolysis. The normal sera and complement were first mixed and after the usual interval amboceptor and corpuscles were added. Table III shows that none of the normal sera except that of the ox acting on its own cells possesses in the fresh state any antilytic action; these sera heated to 50° C. develop sometimes antilytic properties, while a temperature of 60° C. brings out, almost uniformly, this activity. The absence in normal sera of antilytic power may be due to a masking of antagonistic action by interaction of suitable complements and amboceptors, but judging from the effect of the temperature of 50° C. it is more probable that the antilytic principle is present in the sera but is prevented from reacting with complements. If we turn now to a consideration of the action of normal sera in these several states upon a normal serum hæmolysin we shall see a considerable difference of effects. The antilytic action of sheep, ox, and dog sera was studied upon normal dog serum, the complete hæmolytic power of which was for ox corpuscles 0.35 cubic centimeter and for sheep corpuscles 0.25 cubic centimenter. Table IV exhibits the results and is to be compared with Table III.

The different effects brought out in Tables III and IV are considerable. Fresh normal serum of sheep and ox protect their own and each other's corpuscles from lysis by dog serum, but they are able only after heating to 56° C. or higher to protect these corpuscles from lysis by immune sera. In what does this distinction exist? Are the lysins of normal and immune sera distinct and do the normal antilysins act upon one set of lysins only, or are they identical, and do differences in power introduce

a new factor? The fact that heating the normal sera abolishes the differences in effect seems to point to a similarity in nature of the antilysins acting upon normal and immune sera. But in this case the antilysins must exist in different states in

TABLE III.

Immune amboceptor + Complement.	Sheep serum 0.8 c.c.				Dog serum 0.8 c.c.				Ox serum 0.8 c.c			
	Fresh	50° C	60° C	No serum	Fresh	50° C	60° C	No serum	Fresh	50° C	60° C	No serum
Anti-sheep amboceptor 0.1 guinea pig complement 0.02	CH	moderate	none	CH	CH	CH	trace	CH	CH	CH	moderate	CH
Anti-dog amboceptor 0.2 guinea pig complement 0.02	CH	CH	trace	CH	CH	much	none	CH	CH	CH	moderate	CH
Anti-ox amboceptor 0.1 guinea pig complement 0.05	CH	much	slight	CH	CH	CH	none	CH	slight	moderate	CH*	CH

*Slightly quicker than in absence of serum.

TABLE IV

Dog serum and corpuscles.	Sheep serum 1.0 c.c.				Ox serum 1.0 c.c.				Dog serum 1.0 c.c.			
	Fresh	50°C	60C°	No serum	Fresh	50°C	60°C	No serum	Fresh	50°C	60°C	No serum
Dog serum 0.25 vers. Sheep corpuscles	none	none	none	CH	trace	trace	trace	CH	CH	CH	none	CH
Dog serum 0.35 vers. Ox corpuscles	none	none	none	CH	none	trace	CH	CH	CH	CH	trace	CH

the sera through which the two different actions are produced. I conceive that the antilysin exists in combination with other bodies in normal sera, and that part of it is easily extracted from this combination. Another and larger part, possibly, is liberated when the serum is heated to 50° C. All is set free when the temperature is raised to 60° C. The quantity which is readily yielded suffices to neutralize the complement which reacts with the amboceptors of normal sera, but is insufficient to neutralize the complement which reacts with the amboceptors of immune sera. At 50° C. a sufficiency of the antilysin is liberated to neutralize all the complement in the native sera, hence this inactivation; but as it rarely happens that a quantity in excess of this is liberated, sera heated to this temperature tend not to be antilytic. Since at 60° C. all antilysin is rendered available,

the antihæmolytic action is exhibited at its height. Perhaps the spontaneous disappearance of complement is caused by slow liberation of the antilysin in the shed blood.

Another possible explanation should be considered. The complement-antilysin is reversible. In the case of normal serum the degree of reversibility is small; but the large number of amboceptors contained in immune sera may through mass action possess the power of splitting the loose compound and appropriating the complement.

It is desirable to study more clearly the neutralizing action of heated dog serum upon fresh dog serum-hæmolysis of sheep and ox corpuscles. Samples of dog serum were heated respectively to 50°, 56°, 70°, and 90° C. In the case of the last two specimens the serum was diluted with double the volume of 0.9 per cent. salt solution. In the tests which follow guinea-pig complement was employed. The heated serum and complement were first mixed and kept for one hour at 37° C.; the corpuscles were next added, and after another half hour the normal and immune sera were introduced. The total volume was in each test three cubic centimeters, and the final reading was made at the end of two hours standing at 37° C. Table V gives the results, which may be expressed as follows: At 50° C. dog serum does not develop the antagonistic properties; at 56° and 70° C. the serum is rendered highly antagonistic—anticomplementary against the several combinations employed. Heated to 90° C. the serum loses the antihæmolytic action. Since serum complements and amboceptors are destroyed by temperatures of 70° C. the antilytic action here described cannot be explained by supposing the production of complementoid or amboceptoid. The disappearance of anticomplementary action in serum heated to 90° C. may, possibly, be explained by supposing (1) destruction, (2) the formation of a new fixed compound of antilysin and serum constituent, (3) the masking of anticomplementary by auxilytic action. Since, as will be shown later in this paper, I have succeeded in separating in an active state the anticomplementary substance, and since it shows, in this form, marked thermostability, the first supposition may be dismissed. To decide between the last

two possibilities is less easy, but the following facts may be stated: Sera heated to from 80° to 90° C. becomes auxilytic; but, when deprived of lipoids, heating to these temperatures does not bring out this property. The progress of hæmolysis when serum heated to 90° C. is present is slow as compared with the controls, a result that may be accounted for by supposing the interaction of two opposing forces, anticomplementary and auxilytic, which are not absolutely balanced.

Inasmuch as normal dog serum acquires when heated to 56°, or higher, antagonistic properties against the same serum in the fresh state, it is difficult to account for the phenomenon on the basis of complementoid or amboceptoid production.

TABLE V.

	Normal dog serum 0.5 c.c. each.				Control no serum.
	50° C—30″	56° C—30″	70° C—15″	90° C—15″	
Anti-sheep amboceptor (goat) (50° C.) 0.4 guinea pig complement 0.02	CH	none	none	CH slow	CH
Anti-sheep amboceptor (rabbit) (50° C.) 0.1 guinea pig complement 0.01	CH	none	none	CH slow	CH
Anti-ox amboceptor (rabbit) (50° C.) 0.1 guinea pig complement 0.02	CH	none	none	CH slow	CH
Normal dog amboceptor (50° C.) 0.5 guinea pig complement 0.4 guinea pig corpuscles.	CH	slight	none	CH slow	CH
Normal dog amboceptor (50° C.) 0.5 guinea pig complement 0.4 Sheep corpuscles.	CH	trace	slight	CH slow	CH
Normal dog serum 0 25 sheep corpuscles.	CH	trace	trace	CH slow	CH

ABSORPTION OF THE ANTILYSIN BY BLOOD CORPUSCLES.

Since heated serum interferes with hæmolysis when brought into fluid relation with the hæmolytic serum, the question arises whether this action is exerted only against the serum hæmolysin or

whether it influences the corpuscles themselves. The experiments already related show, quite conclusively, that the thermostable antilysin is anticomplementary in effect. It is to be assumed that, under the condition of experiments given, the complement and antilysin combine to form, as far as hæmolysis is concerned, an inert compound. These effects do not indicate whether in the reactions the corpuscles behave in an entirely passive manner. The next experiments to be described show that under certain conditions the corpuscles may take up the antilysin, through the appropriation of which they show an increased resistance to hæmolytic substances.

If blood corpuscles are suspended in serum heated from 56° to 70° C., at 37° C. for several, at room temperatures for eighteen, hours, separated and washed free from serum, they will be found to have become altered in susceptibility to serum hæmolysis. Tables V, VI, VII, and VIII give the results of three such experiments. Thoroughly washed sheep corpuscles, one cubic centimeter, were mixed with four cubic centimeters of the several heated normal sera and kept at 37° C. for six hours. The separated corpuscles were washed with 0.9 per cent. salt solution, thirty cubic centimeters being used at each washing. The corpuscles were then subjected to the action of anti-sheep serum (inactive) and guinea-pig complement (Table VI), or fresh rabbit serum (Table VII), or fresh dog serum (Table VIII).

TABLE VI.

Anti-sheep amboceptor (50°C.) 0.1 c.c.	Normal sera used for digesting sheep corpuscles.			
Quantity of complement	Rabbit No. 11 (60° C.)	Ox serum (60° C.)	Dog serum (60° C.)	Control untreated corpuscles.
0.15 c.c.	much H	CH	moderate H	CH
0.1 "	none	CH	none	CH
0.05 "	none	much H	none	CH
0.03 "	none	slight	none	almost CH
0.02 "	none	none	none	much H

TABLE VII.

Fresh rabbit serum	Normal sera used for digesting sheep corpuscles.			
	Rabbit No. 12 (60° C.)	Ox serum (60° C.)	Dog serum (60° C.)	Control untreated corpuscles
0.5 c.c.	none	CH	CH	CH
0.3 "	none	much	much	CH
0.2 "	none	moderate	moderate	almost CH
0.15 "	none	slight	slight	moderate
0.1 "	none	trace	trace	slight

TABLE VIII.

Fresh dog serum	Normal sera used for digesting sheep corpuscles.			
	Rabbit No. 13 (60° C.)	Rabbit No. 14 (60° C.)	Ox serum (60° C.)	Control untreated corpuscles.
0.2 c.c.	CH	CH	CH	CH
0.1 "	moderate	much	CH	CH
0.07 "	none	none	slight	CH
0.05 "	none	none	slight	much
0.04 "	none	none	trace	slight
0.03 "	none	none	none	trace
0.02 "	none	none	none	none

TABLE IX.

	Temperatures to which the normal dog serum was heated before being employed for digesting corpuscles from the same blood.					
	Fresh	50° C	56° C	70° C	90° C	Untreated corpuscles
Anti-dog amboceptor 0.05 c.c. guinea pig complement 0.02 c.c.	CH	CH	slight H	trace H	slight H.	CH

TABLE X.

Amount of Serum	Normal dog serum (56° C.)		Normal rabbit serum (56° C.)	
	Digested	Not digested	Digested	Not digested
0.5 c.c.	CH	none	CH	none
0.3 "	"	"	"	none
0.2 "	"	"	"	trace
0.1 "	"	trace	"	slight
0.05 "	"	almost CH	"	almost CH
0.03 "	"	CH	"	CH
0.02 "	"	"	"	"
Control, no serum	CH	CH	CH	CH

TABLE XI.

Amount of digested serums.	Dog serum 56° C.			Sheep serum 56° C.			Ox serum 56° C.		
	Rabbit cells	Ox cells	No digestion	Rabbit cells	Ox cells	No digestion	Rabbit cells	Ox cells	No digestion
0.4 c.c.	slight	CH	none	CH	CH	none	much	CH	none
0.3 c.c.	moderate	CH	none	CH	CH	trace	almost H	CH	trace
0.2 c.c.	much	CH	none	CH	CH	slight	CH	CH	slight
0.1 c.c.	CH	CH	trace	CH	CH	much	CH	CH	CH

The tables show that sheep corpuscles treated with several heated sera have their resistance to serum hæmolysis increased and that the effect is overcome by increasing the quantity of complement in the mixture (Table VII) or the quantity of the normal hæmolytic sera (Tables VI and VIII). In these tests the heated sera were foreign to the corpuscles; the next tests were made with serum and corpuscles from the same animal. Table IX shows that if dog corpuscles are digested in heated dog serum they acquire the power of resisting a combination of amboceptor and complement which completely hæmolyzes untreated corpuscles, and, also, that this property of protecting the corpuscles is developed by a temperature of 56° C. but not of 50° C. A second experiment with serum and corpuscles shows that at 56° C. the property is likewise developed in this serum for its own corpuscles.

The evidences which were obtained of the absorption by corpuscles of some substance present in heated serum which interferes with serum hæmolysis would have led naturally to the ascertainment of a corresponding disappearance of the substance from the sera in which the corpuscles were digested. It happened that this phenomenon was observed before the increase in resistance of the treated corpuscles was discovered and the observation led to the discovery of the protection of corpuscles. Table X shows that heated dog and rabbit sera, digested with corpuscles, lose their antihæmolytic property. Two cubic centimeters of dog and rabbit sera were heated to 56° C. and digested with one cubic centimeter of highly condensed (equal to five cubic centimeters original blood) washed sheep corpuscles at

20° C. for eighteen hours. The sera separated from the corpuscles were tested along with the heated sera in which no corpuscles had been placed. Anti-sheep amboceptor (inactive) 0.1 cubic centimeter and guinea-pig complement 0.1 cubic centimeter were used as the hæmolytic combination. Similar experiments to this did not always give uniform results, due doubtless to difference in degree of extractions of the antilytic bodies.

The similarity of the influence of the sheep corpuscles upon the heated rabbit and dog sera in removing antilytic bodies may be taken to indicate that the removal of the antilysin from the sera is not an act of elective absorption, and hence that these antilysins are probably not specific bodies. The experiment summarized in Table XI proves conclusively that any kind of corpuscles can deprive the heated sera of the protective property. Normal dog, sheep, and ox sera were heated to 56° C. and two cubic centimeters of each were treated with one cubic centimeter of condensed rabbit corpuscles. Heated dog and sheep sera were also treated with ox corpuscles. The separated sera were tested for antihæmolytic power.

EXTRACTION OF ANTILYSIN FROM SERUM AND CORPUSCLES.

The next step was to obtain, if possible, this thermostabile anticomplementary substance in a state of greater concentration. The earlier work of Landsteiner and v. Eisler, and Bang and Forssman, and my own observations indicated that the body probably occurred among the lipoid constituents of the blood. It was, therefore, among these that I sought it. The method which I employed does not differ essentially from the extraction method of Bang and Forssmann. I employed the separated serum and corpuscles for my work. Two hundred cubic centimeters of dog and sheep blood were shaken separately with 400 cubic centimeters of ether in a shaking apparatus, and the ethereal extracts separated and dried. These were now extracted with boiling benzol and the residue discarded. The benzol extract was dried and extracted, in turn, with aceton in the cold. Two fractions were obtained: the insoluble fraction consisting chiefly

of lecithin and other phosphatids, and the soluble fraction consisting of cholesterin, fatty acids, neutral fats, and probably other less defined bodies. While the reaction of the insoluble fraction was neutral, the reaction of the soluble fraction was acid. The fractions deprived of aceton were emulsified in forty cubic centimeters each of saline solution. The insoluble fraction exercised no protective or anticomplementary action; hence it was discarded. The soluble fraction, on the other hand, exerted powerful protective activities that were not reduced by filtration through compressed filter paper which removed suspended particles. For the sake of brevity I will speak from now on of this saline extract as "protectin," the effects of which I proceeded to compare with those of the heated sera.

ANTICOMPLEMENTARY AND NON-SPECIFIC ACTIONS OF PROTECTIN.

The next step was, therefore, to expose susceptible blood corpuscles to the action of a hæmolytic combination in the presence of "protectin" from sera and cells and to compare the effects with suitable controls. For the experiments sheep corpuscles, anti-sheep serum (goat, inactivated at 50° C.) 0.2 cubic centimeter and guinea-pig complement 0.1 cubic centimeter were employed. Tables XII, XIII, and XIV show the results, which may be stated as follows: Protectin prevents hæmolysis of sheep corpuscles quite as completely as do heated sera and agrees with the heated sera in failing to exhibit evidences of specific activity. I now proceeded to ascertain whether the action was upon the complement of the hæmolytic combination or upon some other constituent. Table XV gives the results obtained and is taken to indicate action upon the complement of the hæmolytic complex. An identical result was obtained with sheep protectin.

ABSORPTION OF PROTECTIN BY CORPUSCLES.

Since up to this point the protectin solution acted in a manner which was not to be distinguished from the heated sera, a test of the power of corpuscles to absorb the active agent from the

solution was next made. Table XVI shows that in this respect the protectin solution is comparable with the heated sera.

EXTRACTION OF PROTECTIN FROM DRIED AND HEATED SERA AND CORPUSCLES.

There was available for preparing the protectin a large quantity of dried sera and corpuscles. These products had been collected, dried, and subjected for thirty minutes to a temperature of 150° C. I had at my disposal the sera and corpuscles of dog, ox, and horse which, after the treatment just mentioned, had been on hand for nearly two years. The several products were powdered and extracted in a shaker with ether (forty-eight hours), after which the further steps were identical with those already given. I was thus enabled to obtain relatively large quantities of the protectin fraction which was tested against various normal and immune sera with the results already given. By means of many tests I came to the conclusion that both blood serum and corpuscles, of all the mammals studied by me, contain a body which has the property of protecting corpuscles of different mammals from the effects of serum hæmolysins, and that this body is highly stable and very resistent to heat.

TABLE XII.

Amount of protectin in c.c.	Source of protectin			
	Dog		Sheep	
	Serum-protectin	cell-protectin	Serum-protectin	Cell-protectin
0.5	none	none	none	none
0.4			"	
0.3			trace	
0.2	trace		slight	trace
0.15	"		"	"
0.1	slight		much	slight
0.07	moderate	trace	almost CH	"
0.05	almost CH	slight	CH	almost CH
0.03	CH	"		CH
0.02		almost CH		
0 (control)		CH		

TABLE XIII.

Sheep corpuscles were to be protected from fresh active immune serum (goat).

Amount of anti-sheep serum	Control (no protectin)	Sheep cell-protectin 0.2 c.c.
0.3 c.c.	CH	trace
0.2 "		
0.15 "		
0.1 "		
0.07 "	much H	none
0.05 "	slight	
0.04 "	trace	
0.03 "	none	

TABLE XIV.

Sheep corpuscles were to be protected from fresh normal dog serum.

Amount of dog serum	Control (no protectin)	Sheep cell-protectin 0.4 c.c.	Dog cell-protectin 0.4 c.c.
1.0 c.c.		moderate	slight
0.7 "		slight	"
0.5 "			
0.5 "	CH		
0.3 "			
0.2 "			
0.1 "		none	none
0.07 "			
0.05 "	slight		

TABLE XV.

1) Dog serum-protectin 0.3 c.c. + g. p. complement 0.1, 1 hour at 37° C. + anti-sheep amboceptor 0.2 c.c. + corpuscles = no H.
2) Dog serum-protectin 0.3 c.c. + g. p. complement 0.2 c.c., 1 hour at 37° C. + anti-sheep amboceptor 0.2 c.c. + corpuscles = CH.
3) Dog serum-protectin 0.3 + g. p. complement 0.1 c.c., 1 hour at 37° C. + anti-sheep amboceptor 0.4 c.c. + corpuscles = no H.
4) No protectin (control) + g. p. complement 0.1 c.c., 1 hour at 37° C. + anti-sheep amboceptor 0.2 c.c. + corpuscles = CH.

TABLE XVI.

1) Sheep corpuscles 0.5 c.c. + sheep cell-protectin 4.0 c.c. kept 4 hours at 37° C., after which the corpuscles were separated from the fluid and washed in 0.9% saline solution.
This will be called "sheep cell-protectin treatment."
2) Sheep corpuscles 0.5 c.c. + dog cell-protectin 4.0 c.c. kept 4 hours at 37° C.,

after which the corpuscles were separated from the fluid and washed in 0.9% saline solution.

This will be called "dog cell-protectin treatment."

The hæmolytic complex was: anti-sheep amboceptor (50° C.) 0.1 c.c. and guinea pig complement 0.02 c.c.

Observation: 30 minutes at 37° C.

Result: "Sheep cell-protectin treatment" plus hæmolyser give no hæmolysis.
"Dog cell-protectin treatment" plus hæmolyser give slight hæmolysis.
Untreated sheep corpuscles plus hæmolyser give complete hæmolysis.

This condition of thermostability is one of its most interesting features, and brings it, at first sight, into contrast with the thermostabile, so-called protective principles of normal serum

TABLE XVII.

Temperature	Duration of heating	Color	Reaction	Antihæmolytic power
100° C	1 min.	water clear	acid	no H
	5 min.	"	"	"
	15 min.	"	"	"
	30 min.	"	"	"
	60 min.	"	"	"
	90 min.	"	"	trace H
	120 min.	trace yellow	neutral	slight H
120° C	10 min.	light yellow	"	"
135° C	10 min.	light amber	alkaline	moderate H
150° C	10 min.	amber	"	"
unheated			acid	no H

Remarks: To raise the temperature from 100° C. to 120° C. 40 minutes, from 120° to 135° C. 10 minutes, and from 135° to 150° C. 10 minutes are required. To cool the antoclave from 150° to 100° C. it took about 60 minutes.

The question presents itself whether bodies which exhibit such marked differences in properties can possibly be identified with each other. My own views, already outlined on pages 732 and 736, make it possible for me to conceive readily that they are identical. It will be unnecessary to repeat here what I have already stated regarding my reason for thinking that loss of protective property of serum when heated to 90° C. is not due to destruction of the protective body, but to development of auxilytic compounds or recombination of the former body.

My next experiment was to ascertain the effects of high temperatures upon the protectin solution. Dog corpuscle protectin in 0.9 per cent. salt solution was placed in tubes, which were

hermetically sealed. Table XVII shows the temperatures to which the tubes were heated and the alteration in physical appearances which resulted. The antihæmolytic power of the solutions was tested against anti-sheep serum (50° C.) 0.2 cubic centimeter, guinea-pig complement 0.05 cubic centimeter, and sheep corpuscles. The controls give complete hæmolysis. The quantity of the protectin used in each tube was 0.2 cubic centimeter. From the table it will be seen that the solution is highly resistant to heat, but that a diminution in activity, associated with changes in reaction, occurs at the higher temperatures.

SUMMARY.

Blood serum contains normally certain principles which inhibit serum hæmolysis by interfering with the action of complement. In the case of most sera the anticomplementary action appears only after heating to 56° C. or higher.

The inhibiting action of this principle is directed against alien as well as native complements, and is non-specific in character.

It would appear as if the inactivation of serum at 50° C. or thereabouts were due to a partial liberation of the antilytic principle which just about suffices to neutralize the action of the native complement. As the temperature of the serum is raised, up to a certain point, more and more of the antilysin is set free until the serum comes to contain an overplus, in excess of the quantity neutralizing its own complement, which is capable of interfering with the action of additional native or alien complements.

Serum heated to 90° C. is less antilytic than serum heated to 70° C., either because the antilysin has entered into new, more stable compounds which prevent its action, or, as is more probable, because of the liberation from the serum of a second group of principles in themselves hæmolytic directly, or indirectly by increasing the power of serum hæmolysins (auxilysins). This latter action tends to mask and to suppress the inhibitory influences of the antilysin.

The antilysin is removed from serum by digestion with many

kinds of blood corpuscles which, apparently, absorb the principle. While, by reason of this treatment, the serum is deprived of its inhibitory power, the corpuscles have acquired greater resistance to serum hæmolysins.

By treating blood serum and corpuscles with ether the antilysin can be extracted. The ethereal extract, freed from lecithin and certain related bodies, contains the antilysin in a concentrated but not in a pure form, which can now be taken up in saline solution in which it dissolves.

The saline solution of the antilysin, which for convenience has been denominated "protectin," behaves in all respects like the native antilytic sera except that it is uninfluenced by temperatures of 90° C. or even higher temperatures. Protectin inhibits serum hæmolysis directly by neutralizing complement and indirectly after absorption by corpuscles by increasing their resistance. Hence it is highly probable that the antilytic principle of heated serum and protectin extracted from serum and corpuscles are the same substance.

Thermostability is one of the characteristic properties of protectin. Serum and corpuscles, first dried, may be heated to 150° C. without losing the protecting principle, and the principle persists in such heated products for at least two years. Protectin in solution is unaffected by a temperature of 100° C. maintained for one hour, and suffers only slight reduction in activity at the expiration of two hours; while temperatures ranging from 135° to 150° C. bring about marked reduction in protective power. As these alterations are produced by high temperatures the reaction of the solution changes from acid, through neutral, to alkaline.

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